THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. VIII OCTOBER, 1927 No. 5

COAGULATION TIME OF BLOOD DURING ANÆSTHESIA.

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Received for publication May 30th, 1927.

IN an attempt to elucidate the causes of pulmonary embolism following operation, it was decided to investigate the coagulation time of blood during the operation. Several studies of the changes after operation have already been published. Bolognesi (1919) always found an increased coagulability after operation independent of the administration of an anæsthetic. Chauvin and Esmenard (1923) found modification of small magnitude and entirely within the limits of experimental error in the clotting time after operations under various anæsthetics. Mendenhall (1915) investigated the problem on decerebrate cats, and found an average decrease of 15·2% in the coagulation time on administration of 50 c.c. of ether. He found no diminution after removal of the adrenal glands, and attributed the changes observed to the liberation of adrenaline. Nolf (1920) investigated the action of ether and chloroform on plasma, and found they had a definite thromboplastic action, so that a direct action of the blood may be responsible.

It was found in the course of this work that closed ether anæsthesia diminished the coagulation time of blood. The shot coagulometer method of Dale and Laidlaw (1911–1912) was used to determine the coagulation time, as it was found to give very accurate results, the mean average error being less than 5 secs. The cases were all operated on in the afternoon, and the pre-operative determinations were made between 12 a.m. and 1 p.m. (in a few cases between 11 a.m. and 12 a.m.), one or two readings being taken. During the operation several readings were taken in all cases. Fifty patients operated on by Mr. Braithwaite and Mr. Moir, to whom my greatest thanks are due, were used, the investigations covering the period September, 1926, to March, 1927. As it was found that the type or length of operation bore no relation to the effect on the coagulation time, the cases are not quoted individually. The operations include appendicectomy, gastro-enterostomy, cholecystectomy, radical cure for hernia, castration, hydrocele operations, splenectomy, cholecyts-gastrostomy, operation for hæmorrhoids, removal of ovarian cyst, Rodman's

operation, and removal of band in intestinal obstruction (the only acute case in the series).

TABLE I.

Percentage diminution in coagulation time.	Number of cases.	Percentage of cases.
– 5–5	9	18
6–10	6	12
11–20	23	46
21–30	9	18
31–40	3	6

Table I gives the results obtained. As the average coagulation time is not far from 100 seconds, the percentage diminution is very nearly similar to the actual diminution in seconds. It is seen that in 20% of cases no change was observed. In all the other cases an increased coagulability was found. A reduction of 11–15% most commonly took place, but it is important to note that 6 cases showed a reduction of 25–40%. (Two cases actually diminished 40 seconds from 120 and 108 seconds respectively.) As a rule the maximum diminution was reached within 15 minutes of the beginning of the anæsthesia, and determinations made whilst the initial incision was being made were the same as those observed at the end of the operation. In about 20% of cases, however, the maximum fall was not observed at first, and the coagulability actually increased during the course of the operation. The duration of this change has not been investigated, but in 7 cases in which the coagulation time was taken on the day after the operation, the normal time had only been reached in 3. Bolognesi (1919) found that the diminution lasted several days in all his cases. Dale and Laidlaw (1911–12), who investigated the normal coagulation time, found there was no diurnal variation, and the above changes must therefore be related to the operation. That they are due to the anæsthesia arises from the fact that in the majority of cases the maximum fall was observed in the initial stages of the operation.

To prove this point more fully it was decided to institute further experiments, viz. to investigate the effect of subcutaneous injection of ether on the coagulation time. All the experiments were performed in the afternoon between 2 p.m. and 3 p.m., on patients who had not yet been operated on. The coagulation time was determined; then 3 c.c. of ether were injected subcutaneously in the forearm, and the coagulation time was again taken after a certain interval.

Table II shows the results obtained. It is seen that in a majority of cases there is a definite fall. It is not so great, and does not involve such a high percentage of cases as in anæsthesia, but this is probably due to the smaller concentration of ether reached in the blood.

The question arises whether the changes observed take any part in the causation of post-operative pulmonary embolism, and although it seems logically probable that an increased coagulability of the blood, whatever be its causation, would tend to increase the tendency to thrombosis, it is impossible to give an absolute answer. It may be worth mentioning that one of the cases investigated died from pulmonary embolism (confirmed by post-mortem

examination) seven days after a Rodman operation for carcinoma of the breast. Her coagulation time was 95 seconds on the morning of the operation, and diminished to 94 seconds during anæsthesia. In view of a recent statistical study of Lister (1927), in which the importance of age and abdominal incision in the ætiology of pulmonary embolism is clearly brought out, it seemed worth while tabulating the results obtained in age-groups, and also dividing the cases into abdominal and non-abdominal.

TABLE II.

Patient.	Sex.	Age.	Coagulation time. Before.	Coagulation time. After.	Interval.
1. A. J—	M.	22	108 secs.	87 secs.	60 mins.
2. J. B—	M.	68	97 ,,	94 ,,	70 ,,
3. T. P—	M.	46	80 ,,	82 ,,	40 ,,
4. W. M—	M.	41	93 ,,	60 ,,	65 ,,
5. H. T—	M.	63	83 ,,	77 ,,	65 ,,
6. F. P—	M.	23	78 ,,	65 ,,	45 ,,
7. T. R—	M.	50	107 ,,	85 ,,	35 ,,
8. T. S—	M.	64	94 ,,	87 ,,	40 ,,
9. T. S—	M.	23	88 ,,	78 ,,	30 ,,
10. S. T. R—	M.	23	100 ,,	102 ,,	35 ,,
11. T. G—	M.	21	95 ,,	85 ,,	35 ,,
12. W. T—	M.	29	85 ,,	75 ,,	30 ,,
13. H. L—	M.	41	98 ,,	85 ,,	35 ,,
14. V. N—	M.	29	92 ,,	79 ,,	30 ,,
15. F. W—	M.	16	92 ,,	77 ,,	30 ,,

TABLE III.

Age of patient.	Number of cases.	Average coagulation time.	Average diminution during operation.
10–20	1	102 secs.	12 secs.
21–30	6	97 ,,	11 ,,
31–40	13	98·5 ,,	17·5 ,,
41–50	16	94 ,,	16 ,,
51–60	9	91 ,,	11 ,,
61–70	5	89 ,,	7 ,,

From Table III it can be seen that there is a slight diminution of the average coagulation time with advancing years. The average diminution is smallest over 60 years, but is fairly constant below that. It seems therefore probable that changes in the coagulation time are not responsible for the much greater embolism incidence in the higher age-groups.

TABLE IV.

	Number of cases.	Average diminution.
Abdominal	41	13 secs.
Non-abdominal	9	19 ,,

Table IV shows the average diminution in operations involving the opening of the abdomen and those which do not, and clearly shows that the clotting time of the blood observed is not responsible for the greater number of cases of embolism following the former.

SUMMARY.

1. In operations under closed ether anæsthesia there is a diminution in the coagulation time of blood in 80% of cases investigated, reaching up to 40% of the initial coagulation time.

2. The diminution is already present during the initial stages of the operation in 75% of cases, and seems to be independent of the type of operation or duration of the anæsthesia.

3. Subcutaneous injection of ether is followed in a majority of cases by an increased coagulability of the blood within half an hour.

4. The coagulation time diminishes slightly with advancing age, within the limits of the data obtained.

5. The increased coagulability does not increase with advancing age, and is not greater in cases involving the opening of the abdomen.

I should like to express my sincerest thanks to Mr. L. R. Braithwaite, Director of Surgical Research at Leeds University, for his advice and encouragement given me in this work.

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NOTE ON SOME PATHOLOGICAL CHANGES IN THE TISSUES DURING ATTEMPTED ACCLIMATIZATION TO ALTERATIONS OF O_2-PRESSURE IN THE AIR.

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Received for publication July 6th, 1927.

DURING some physiological experiments in which animals were exposed for prolonged periods to altered O_2-pressures in the air, with the view to studying problems of acclimatization, the tissues were found to exhibit the pathological changes described below.

Outline of Experiments.

The outline of the experiments is given in Table 1; details of the technique are recorded in recent papers (Campbell, 1927). With the aid of three different chambers, the animals—cats, monkeys, rabbits, cavies, rats and mice—were exposed for considerable periods to abnormal O_2-pressures in the air, with temperature about 15–20° C. In experiments Nos. 1 to 8 the barometric pressure was normal, the O_2-pressure being altered; in experiment No. 9 the barometric pressure was gradually lowered as well as the O_2-pressure. Changes in barometric pressure are of little significance under the conditions specified, the alterations in O_2-pressure being the important factor.

In experiments Nos. 1 to 5, the O_2-pressure was increased to a maximum about 200% above normal—that is from about 150 mm. Hg. to 420 mm. Hg., after allowing for water vapour. Experiments Nos. 6 to 9 dealt with lowered O_2-pressure in the air, the minimum being about 60% below normal or about 50 mm. Hg., equivalent to that which exists at an altitude of about 30,000 feet.

LOW O_2-PRESSURE EFFECTS.

Symptoms.—The animals tested differed markedly in their powers to tolerate prolonged exposure to O_2 at low pressure. Some rabbits, rats and mice seemed most resistant; judging from appetite and weight they appeared to have become acclimatized to O_2-pressure at about 10% of an atmosphere (70 mm. Hg.), equivalent to that of an altitude of 20,000 ft. After a certain degree of acclimatization they were able to exist for at least 7 days' continuous exposure to O_2-pressure at about 7% of an atmosphere (50 mm. Hg.), equivalent to that at 30,000 ft. Under the latter conditions they were deteriorating rapidly. Cats, cavies and a monkey tested exhibited very little

ability to tolerate low O_2-pressure, suffering great loss of appetite and weight under O_2-pressure at 10% of an atmosphere. The chief symptoms observed in all animals affected adversely were drowsiness, weakness, loss of appetite and fall of O_2-consumption; such symptoms suggest the onset of heart failure.

TABLE I.—*Outline of Experiments.*

Experiment reference number.	Animals employed.	Barometer mm. Hg.	Average O_2-pressure in inspired air. mm. Hg.	Average O_2-pressure in inspired air. %	Approximate altitude corresponding to O_2-pressure (feet).	Duration (days).
1	2 cavies, Nos. I and II	745	141	20·1	Sea-level	8
		,,	330	47·1	...	24
		,,	433	61·9	...	33
		,,	141	20·1	Sea-level	14
2	1 cat, No. II	,,	140	20·0	,,	7
		,,	287	41·0	...	16
3	3 cats, Nos. I, II and III	,,	326	46·6	...	11
	3 cats, Nos. I, III and IV	,,	376	53·7	...	7
		,,	140	20·0	Sea-level	24
4	6 rats and 6 mice	,,	140	20·0	,,	10
		,,	297	42·4	...	14
		,,	424	60·6	...	21
5	2 monkeys, Nos. I and II	,,	138	19·7	Sea-level	6
		,,	311	44·4	...	18
		,,	371	53·0	...	11
		,,	140	20·0	Sea-level	14
6	2 cavies, Nos. I and II	,,	82	11·7	15,000	10
		,,	72	10·3	20,000	7
		,,	67	9·5	22,500	7
7	3 cats, Nos. I, III and IV	,,	141	20·1	Sea-level	6
		,,	86	12·3	15,000	14
		,,	64	9·1	25,000	15
8	1 monkey, No. II	,,	137	19·6	Sea-level	4
		,,	95	13·6	13,000	12
		,,	76	10·9	18,000	7
		,,	70	10·0	20,000	6
		,,	147	21·0	Sea-level	20
9	6 rabbits, 6 rats, 6 cavies, 12 mice	600	116	16·6	7,000	3
		440	85	12·1	15,000	7
		400	77	11·0	18,000	1
		375	72	10·3	20,000	6
		320	62	8·8	25,000	7
		290	56	8·0	27,500	1
		260	50	7·1	30,000	8

Post-mortem examination of the organs of 2 cats (Exp. No. 7), 1 rabbit (Exp. No. 9), 7 cavies (Exps. Nos. 6 and 9) and 5 mice (Exp. No. 9) indicated a general state of vascular congestion and heart failure as the constant feature. All the above animals died except one of the cavies which was killed for observation; where necessary organs from control animals were studied.

Heart.—When examined soon after death the heart was found to be flabby and dilated. Microscopic examination revealed fatty degeneration of the muscle-fibre, in varying degree, similar to that described for cavies during much shorter experiments carried out by Rosin (1926). The degeneration he described was well demonstrated, but one of my cavies (No. I, Exp. No. 6) showed an extreme fatty change much greater—so far as I know—than anything described previously for heart muscle under any condition. An illustration of the heart in question is given in Fig. 1; the animal was living with its heart in this state, being killed for post-mortem examination.

The other animals showed a similar change but of less intensity, the rabbit and the cavies exhibiting the condition in more marked degree than the cats, whose heart muscle revealed only a fine granular form of the degeneration. When the fat was dissolved out the characteristic vacuolation was particularly well marked in the cavies and in the rabbit. Œdema and hæmorrhages were observed in the heart substance of this rabbit (Exp. No. 9). In most of the animals the vessels of the heart were engorged with blood and the muscle striations poorly represented.

Lungs.—Marked congestion of the lungs was present in all animals; sometimes there were hæmorrhages and excess of blood-pigment in the tissues. In the cats the changes resembled those of typical chronic congestion, the interalveolar septa (Fig. 2) being much thickened by plain muscle-fibre; in the case of the cats and mice the lungs were more affected by the congestion than were the other organs. In the cats the tunica muscularis (Fig. 2) of the pulmonary vessels, particularly the arteries, was hypertrophied. Control cats did not show such a condition, but a somewhat similar state was noted in a cat with broncho-pneumonia. Although the other animals, *i. e.* rabbit, cavies and mice, under low O_2-pressure did not exhibit such hypertrophy of the tunica muscularis, perhaps the change in the cats indicated a physiological adaptation to increased pressure in the pulmonary circulation, or the hypertrophy may have aided the onward flow of blood through the lung by increased rhythmical contraction. The rabbit's lungs were almost completely collapsed owing to the presence of fluid in the pleural cavity. Portions of the lungs of most of the animals were emphysematous owing to hyperpnœa caused by O_2-deficiency.

Liver.—Congestion of the liver was observed in all animals, being particularly well marked in the rabbit and the cavies, where the "nutmeg" condition (Fig. 3) typical of heart failure was present. The cells in the centre of the lobules were atrophied and necrosed whilst the vessels were greatly dilated. Very marked fatty degeneration was noted in the rabbit and cavies; a similar degeneration existed in the other animals but in less degree.

Kidney.—Congestion was the only definite abnormality noted in the kidney, although in some cases there did appear to be some atrophy, and also some degree of fatty degeneration in excess of normal.

Spleen.—In some animals there was an excess of altered blood-pigment—additional evidence of the general state of congestion.

Suprarenal.—Some cells of the medulla were necrosed in one of the cats and one of the cavies. Most of the animals showed a loss of cortical lipoid owing to general wasting of tissue. There was also congestion.

Nervous system.—No definite degeneration was detected in the cells of the brain; in some the Nissl granules were not prominent, but in others they were quite normal. The cells of the medullary centres in the region of respiratory control were examined especially, and the nerve-cells showed little change from normal.

In conclusion, it may be stated that congestion of the organs was the outstanding feature; in addition there was fatty degeneration such as is found in cardiac failure and in anæmias.

HIGH O_2-PRESSURE EFFECTS.

Lorraine Smith (1899) proved that animals die from a type of pneumonia if exposed for any length of time to O_2-pressure at or above 70% of an atmosphere (490 mm. Hg.). At present we are considering prolonged exposures to O_2-pressure rather lower than this, viz. 60% of an atmosphere. I (Campbell, 1927) have observed that rabbits, monkeys (Exp. No. 5), cavies (Exp. No. 1), rats (Exp. No. 4) and mice (Exp. No. 4) can acclimatize fairly well to prolonged exposures to O_2-pressure at 60% (420 mm. Hg.) which represents an increase of about 200% above normal. About the same time Barach (1926) obtained similar results, using rabbits only. On the other hand, I have noted a striking difference with cats (Exps. Nos. 2 and 3), which could not tolerate O_2-pressure increased to such a degree, being adversely affected by O_2-pressure even at 40% of an atmosphere. They lost appetite, becoming much weakened and decreasing rapidly in weight. There was no change of body temperature until the O_2-consumption fell markedly; then the temperature fell definitely also. There was no infection in the blood, and post-mortem examination revealed no striking changes in the organs beyond general wasting of tissue and collapse and congestion of some of the alveoli of the lung with the presence of a few catarrhal cells; but there was no sign really typical of pneumonia. Although there was no definite evidence, it might be conceived that some "poison" was formed by the action of O_2 at high pressure upon the lung epithelium, and general poisoning resulted; the cats for some reason could not antagonize this "poison" so well as rabbits, monkeys, cavies, rats and mice; perhaps the diet was responsible. O_2 at very high pressures (+ several atmospheres) was shown by Bert (1878) to cause convulsions; perhaps these are due to the same "poison" suggested above, but acting in acute degree. These theories require confirmation from further research.

SUMMARY.

(1) The pathological evidence from the organs examined indicates that heart failure is the most constant feature in the animals affected adversely by prolonged exposure to low O_2-pressure in the air.

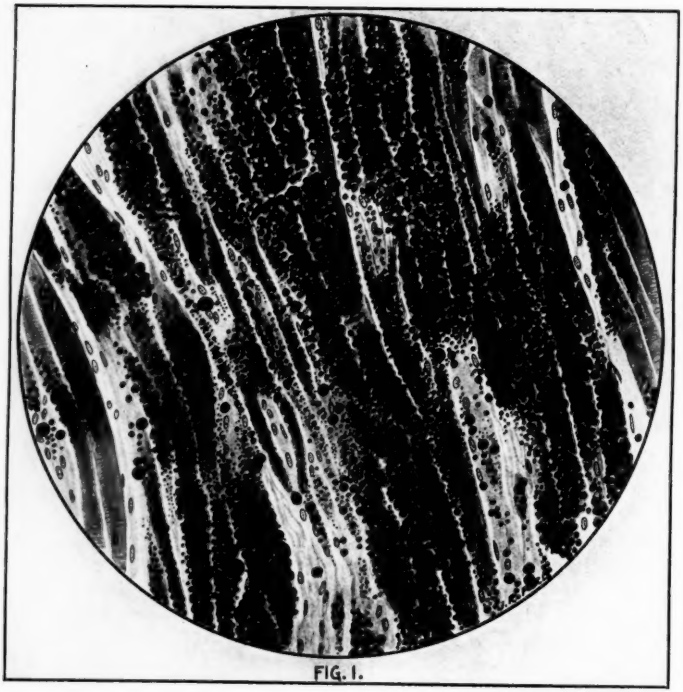

FIG. 1.

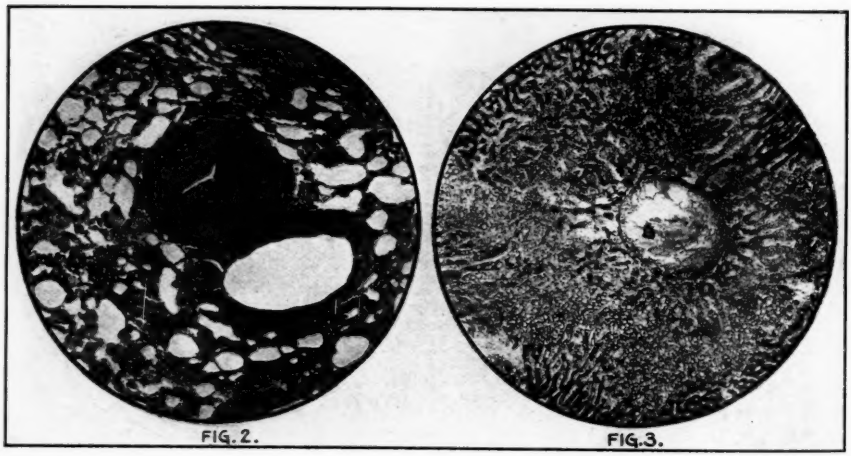

FIG. 2. FIG. 3.

(2) A striking degree of fatty degeneration of the heart was observed in a cavy under low O_2-pressure.

(3) Unlike most other animals cats cannot tolerate for prolonged periods O_2-pressure increased to 60 % of an atmosphere; some general "poisoning" effect due to the action of O_2 upon the lung epithelium is suspected, although the lung itself showed only some collapse and congestion of the alveoli.

I am indebted to Dr. Leonard Hill, and to R. H. Davis, Esq., Managing Director of Messrs. Siebe Gorman, London, for facilities in the performance of the above experiments. Thanks are also due to Capt. S. R. Douglas and Dr. P. P. Laidlaw for advice *re* the pathological changes; and to Messrs. F. Thatcher and G. Probent, the engineers who attended day and night to the decompression chamber at Messrs. Siebe Gorman's premises.

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DESCRIPTION OF PLATE.

Fig. 1.—Monochrome drawing of heart muscle of cavy, showing extreme degree of fatty degeneration during exposure to low O_2-pressure. Scharlach R and Hæmatoxylin. × 156.

Fig. 2.—Effect of low O_2-pressure. Lung of cat No. 3 (Exp. No. 7), showing congestion, increase of plain muscle in the interalveolar septa and hypertrophy of the tunica muscularis of branch of pulmonary artery. Note also small bronchiole. Hæmatoxylin and eosin. × 67.

Fig. 3.—Effect of low O_2-pressure. Lobule of liver of rabbit (Exp. No. 9), showing extreme degree of "nutmeg" condition. Hæmatoxylin and eosin. × 67.

ON SYSTEMIC FACTORS IN THE GENESIS OF TAR-CANCER.

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Received for publication July 11th, 1927.

THE problem to be dealt with in this paper is: Has tar in addition to its local carcinogenetic factors also systemic factors which influence the occurrence of tumours in susceptible animals?

The results of the different investigators who have attacked the question are conflicting, and an additional attempt is made from a somewhat new point of view.

Bang (1925) has shown that if the skin of white mice is thermocauterized cancer can develop in the scar, but the tumour-incidence is rather low. In several series, including 54 animals, he obtained two tumours, one "carcinome au debut," and one "carcinome spino-cellulaire squirrheux."

If tar-painting increases a possible tendency, or diminishes a possible resistance to tumour production, one might find that animals under the influence of tar, when thermocauterized, more readily produce tumours than untarred controls.

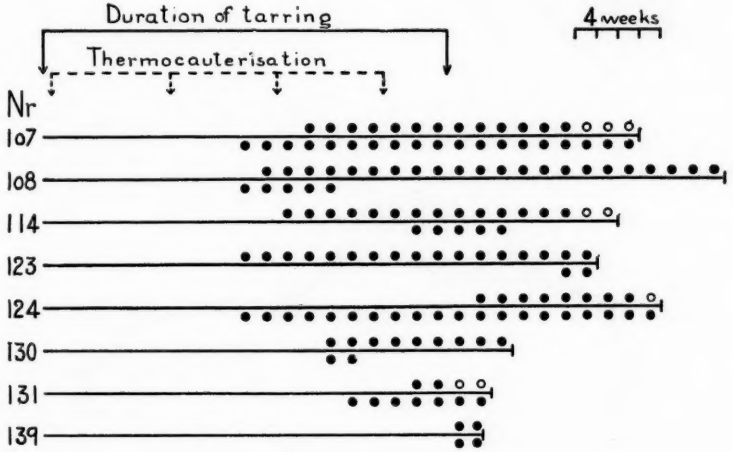
In the experiments to be reported here 50 white mice have been used, 40 males and 10 females; as controls were 40 males. All the animals were 3 months old, belonging to the same strain, and living under identical conditions. The sexes were kept separate.

The first lot was tarred in the *right flank* over an area of 2 square centimetres every second day from September 9th, 1926, to January 16th, 1927. They were superficially thermocauterized with red-hot iron in the *left flank* on September 13th, October 21st, November 28th and December 31st, 1926. If a tumour appeared in the thermocauterized area the operation was not repeated, but the tarring on the other side was continued. The controls were thermocauterized in the same way and on the same days, 20 in one flank, and 20 in both.

The table records the alterations of *the painted area* at the death of the animals. A tumour has been considered malignant only if the panniculus carnosus or nerve-sheaths were invaded or metastases found in distant organs. The table shows that the tar was sufficiently potent:

No tumour.	Warts and papillomata.	Cancer.
5	12	33

In 8 animals tumours were also found in the *thermocauterized area*. The chart gives the different data:



At the top is recorded the duration of tarring and the time of thermocauterization. The line running from each identification number tells how long a time the animal was alive after the experiment began. The dot above the line indicates tumour in the painted area, the dot below, tumour in the thermocauterized area. Black indicates papilloma or wart, ring indicates carcinoma.

As will be seen, 3 of the tumours disappeared again. The remaining 5 were examined in serial sections, and their histological pictures are given in Figs. 1–5. The heavy black line means epithelium, the cross-hatched area means panniculus carnosus.

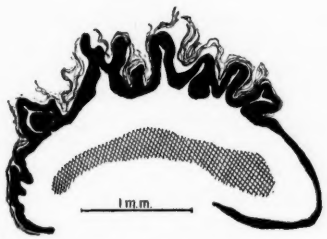


Fig. 1 (No. 107).

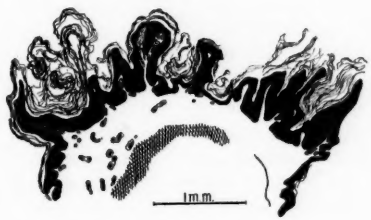


Fig. 2 (No. 123).

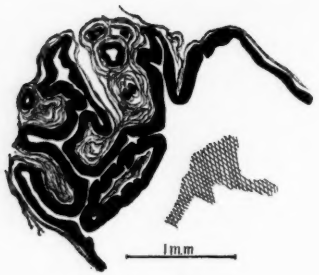


FIG. 3 (No. 124).

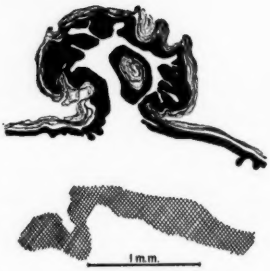


FIG. 4 (No. 139).

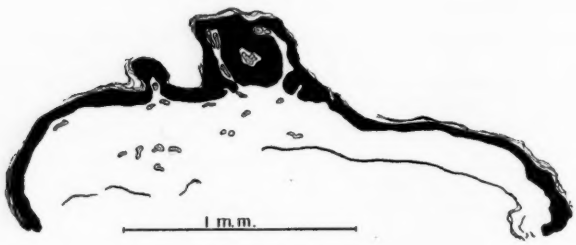


FIG. 5 (No. 131).

At the death of the last of the tarred animals 36 of the controls were alive, and 12 weeks later 34 were still living. Not in a single instance did the controls show any tumour in the thermocauterized area. (In one of the controls a squamous-cell carcinoma developed spontaneously at the base of the right ear.)

The results indicate that the tar, directly or indirectly, through a systemic factor, influences the skin of white mice, with the result that the tumour-incidence after a certain local insult is increased.

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A NOTE ON THE PHYSIOLOGICAL ASPECTS OF INTRA-PERITONEAL BLOOD TRANSFUSION.

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Received for publication July 17th, 1927.

In 1862 von Recklinghausen showed that absorption of defibrinated blood took place from the peritoneal cavity, and suggested that the red blood-cells passed through stomata in the peritoneum.

In 1875 Ponfick showed an increase in the red-cell count following this absorption.

William Hunter (1886) found that 32 to 92% of the total amount of the animal's own blood could be injected intra-peritoneally without ill-effects. He found that there was a slight rise in the blood-count, with a fall on the second day and subsequently a more permanent rise.

Starling and Tubby (1894) injected indigo carmine and methylene blue into the pleural and peritoneal cavities of animals and recovered the pigment in the urine before they could demonstrate it in the lymph. They stated that crystalloids diffused straight into the blood-stream and were not absorbed *viâ* the lymph—anyway in the early stages.

Starling (1898) repeated his experiments and confirmed his previous results. He found absorption from the pleural cavity to be much slower than absorption from the peritoneal cavity.

Buxton and Torrey (1906) demonstrated that after the injection of foreign particles, *i. e.* hen's red blood-cells, typhoid bacilli and lamp-black intra-peritoneally, the lymphatics of the diaphragm were rapidly filled with the injected particles. From there they passed to the anterior mediastinal glands and thence to the thoracic duct. This took place very rapidly, and phago-cytosis in the early stages was not in evidence.

Stomata were believed to exist in the peritoneal cavity till Ranvier (1875) stated definitely that they did not, this latter statement being confirmed by many observers, notably by McCallum (1903), who was unable to find openings, and considered that granules in leaving the peritoneal cavity found their way between the cells. He considered that Ludwig had proved that movement of the diaphragm was a factor in causing absorption of particles. More recent

observers state that particles pass between or through the endothelial cells, but all think that the diaphragm is the main factor causing absorption.

It has been suggested by Bolton that the particles are aspirated from the peritoneal cavity into the lymphatics of the diaphragm by the lymph-currents set up by the respiratory movements. Other observers state that the particles are rubbed through the peritoneal endothelium by the respiratory action of the diaphragm on the liver, and pushed thence into the diaphragmatic lymphatics.

Cunningham (1923), working with fœtal cats, stated that there were no stomata present in their peritoneal cavities.

Siperstein (1923) reviewed the literature in great detail. He advocated intra-peritoneal transfusion as a therapeutic measure, and stated that citrated blood in the peritoneal cavity did not clot.

The present research was undertaken to demonstrate—

(1) The rate of absorption of blood from the peritoneal cavity.

(2) The life-history of the transfused blood before and after absorption from the peritoneal cavity, and the condition of the peritoneum before and after the transfusion.

(3) The mode of absorption of the red blood-cells and their path from the peritoneal cavity into the systemic circulation.

EXPERIMENTAL PROCEDURE.

In the first experiments citrated hen's blood was injected into the peritoneal cavities of guinea-pigs, and films were made of blood taken from the guinea-pigs' ears at intervals following the intra-peritoneal injection. The films were stained by Leishmann's method, and nucleated avian cells were searched for in them. The bird's blood was obtained by venepuncture of the brachio-cephalic vein, and was aspirated into a syringe containing a 3·8% solution of sterile sodium citrate. The citrate solution is isotonic with the blood and was used in the ratio of 1 c.c. of solution to 5 c.c. of blood. The blood, was injected into the lower quadrant of the abdomen of the guinea-pig in the mid-lateral plane with the same syringe. In the earlier experiments the hen's blood was injected into the peritoneal cavities of guinea-pigs in the ratio of 10 c.c. of citrated blood to 450 gm. body-weight of the animal. This is the quantity which Marriott recommends should be given in the case of human infants if the method be employed:

Guinea-pig 1, weight 390 gm., received 9 c.c. of citrated hen's blood.
Guinea-pig 2, „ 400 „ „ 10 „ „ „

The results of examination of ear-vein blood at 15-minute intervals were as follows:

TABLE I.

Minutes after the transfusion.	Guinea-pig 1.	Guinea-pig 2.
15, 30, 45, 60, 75 and 90	*Nil*	*Nil.*
105	Large hyaline cells seen with nucleus of red corpuscle in them.	„

Guinea-pig 1 died from natural causes 21 days later. The peritoneum was found to be glistening. There was neither free fluid nor clot. There was no evidence of adhesions.

Guinea-pig 2 was killed. The peritoneum was found to be normal and to contain no clot.

It is important to test the compatibility of the bloods before giving the transfusion. This was done by incubating the corpuscles of the donor, *i. e.* the hen, with the serum of the recipient, the guinea-pig, and examining the mixture at the end of half an hour for evidence of hæmolysis. If this precaution were omitted and an incompatible blood were given the recipient became very shocked and passed a blood-stained urine. No animal, however, died. No avian cells were seen in the peripheral circulation of the animals so affected. This suggests that there are blood-groups among the lower animals similar to those dividing man.

In the above experiments, although one or two nucleated red cells were seen to be engulfed by large mononuclears, no free nucleated cells were demonstrable in the peripheral circulation of the guinea-pig. We may conclude, therefore, that the guinea-pig's peritoneum is not freely permeable to hen's blood.

Citrated pigeon's blood was next injected into the peritoneal cavities of rabbits, and samples of blood were taken from the rabbits' ears every 15 minutes subsequently.

Rabbit 1, weight 1200 gm., received 10 c.c. of blood.
Rabbit 2, „ 500 „ „ 8 „ „
Rabbit 3, „ 400 „ „ 5 „ „

The results of the examination of blood taken from the peripheral circulation at 15-minute intervals were as follows:

TABLE II.

Minutes after the injection.	Rabbit 1.	Rabbit 2.	Rabbit 3.
15, 30, 45, 60, 75 and 90	+	+	+
105	*Nil*	*Nil*	*Nil.*

Rabbit 2 killed 15 days later. The peritoneal cavity was found to be free from blood and clot. There were no adhesions present and in appearance the peritoneum was normal.

Rabbit 3 killed. The peritoneal cavity was found to be normal.

Nucleated red cells were seen in the peripheral circulation after 15 minutes, and the cells persisted in the peripheral circulation for 48–56 hours after their first appearance, but were never demonstrable in large numbers. Rabbits and fowls were found, however, to be inconvenient with which to work; accordingly mice and pigeons were substituted for them.

A series of mice was given intra-peritoneal injections of citrated pigeon's blood in the ratio of 10 c.c. per 450 gm. of body-weight, and the animals were killed at intervals by means of coal-gas. Smears of blood from the heart were examined and the peritoneal cavity was searched for the presence of free fluid and clots:

TABLE III.

Mouse.	Time in hours.	Smears from heart.	Blood in peritoneum.	Clots.	Peritoneum.
1	6	No cells seen	++++	No	*Nil.*
2	10	Many cells seen ++	++	„	„
3	22	Many cells seen ++++	+	Clot	„
4	34	Many cells seen +++	Thin fluid	„	„
5	46	Fewer cells seen ++	Little serous fluid	Small clot	„
6	80	*Nil*	„ „	„	„
7	118	„	*Nil*	*Nil*	Normal.
1*a*	3	Few cells seen +	++++	No	*Nil.*
2*a*	9	Many cells seen +++	+++	Clots	„
3*a*	50	Few cells seen +	No	No	*Nil.*
4*a*	70	*Nil*	„	*Nil*	Normal.
1*b*	15 mins.	„	++++	Fluid	*Nil.*
2*b*	30 „	„	++++	„	„
3*b*	1 hour	Few cells seen +	++++	„	„

The above experiments show that mice rapidly absorb blood that has been placed in their peritoneal cavities, and that the foreign cells persist in the peripheral circulation for at least 46 hours. There were clots in the peritoneal cavities of the mice killed between the 22nd and the 46th hours following the intra-peritoneal injection. Although clots were formed, no evidence of their becoming organized has been discovered, nor have there been, in any instance, any intra-peritoneal adhesions. It is only reasonable to suppose that the citrate, being a crystalloid, diffuses into the general circulation more quickly than the colloid blood; as the concentration of the citrate in the transfused blood falls, it clots. On this point I am at variance with Siperstein, who states that if the solution of citrate is fresh, clotting does not occur.

The clots that are formed are not very firm, and are eventually readily absorbed, as no transfused blood was found in the peritoneal cavity after the 46th hour.

In view of the fact that clots were formed following a citrate transfusion, defibrinated blood was next injected into a fresh series of mice, and it was found to be absorbed readily, and to leave no clot.

TABLE IV.

Mouse.	Time.	Peritoneum.
1	24	Fluid blood in peritoneum.
2	48	No fluid in peritoneum; no adhesions.

In all the above experiments the recipient of the intra-peritoneal transfusion was given heterogeneous blood. A series of mice was therefore given transfusions of autogenous blood, *i.e.* mouse-blood:

TABLE V.

Mouse.	Time in hours.	Peritoneum.
1	24	Blood.
2	48	*Nil.* Peritoneum normal.

It is clear that mice absorb mouse-blood from their peritoneal cavities with great ease. In this series the peritoneum was clear after 48 hours.

As the peritoneum appeared to be as permeable to cells as to serum, it was decided to estimate the permeability of other serous membranes, and accordingly 10 c.c. of blood were injected into the pleural cavities of each of two rabbits weighing 450 gm. The rabbits were X-rayed in the hope that the rate of absorption of the fluid from the pleural cavity might be visualized. This, however, was not successful, as the free fluid could not be demonstrated radiographically. The rabbits did not tolerate blood in their pleural cavities nearly so well as they did in their peritoneal cavities. No nucleated cells were demonstrable in the peripheral circulation of these animals, and when killed 100 days from the date of the initial transfusion, old blood-clots were found in the pleural cavities of each.

An intra-peritoneal transfusion was next attempted on a hæmophilic boy weighing 40 kgm. The child had a large hæmarthrosis. After being cross-grouped with his father, 400 c.c. of the father's blood was aspirated from one of the brachial veins. A small needle, which was attached to a tube and funnel, was inserted into the child's abdomen in the mid-lateral plane midway between the umbilicus and the anterior superior spine, and the blood slowly run in. After 300 c.c. of the blood had been given, the child complained of a feeling of nausea and vomited. The transfusion was then stopped. The child's coagulation before the transfusion was 4 mins. 15 secs. (method of Dale and Laidlaw) at 37°C.; 48 hours later it had fallen to 1 min. 30 secs. The blood-cells were counted before and after the transfusion.

There was an initial rise in the blood-count followed by a fall and then a more permanent rise. These findings agree with those of William Hunter already quoted. This child was observed for a period of two months after the transfusion and no untoward symptoms arose. The hæmarthrosis gradually subsided.

In the course of the above experiments, although intra-peritoneal punctures were made in a large series of animals in no instance was the bowel perforated, nor was any fluid injected into the lumen of the bowel. In order to ascertain the possibilities of perforating the intestines by puncturing the anterior abdominal wall with a needle similar to that used in giving an intra-peritoneal transfusion, injections of carmine were made into the peritoneal cavities of a series of cadavers. A Wassermann needle and Record syringe were used for these experiments, and in all about 40 punctures were made. In every instance the carmine was found to be in the peritoneal cavity and outside the bowel. Although the lumen of the intestine was searched carefully for the presence of a red stain, in no instance was one found. Serial sections were

made from the diaphragms of many of the mice killed in the above experiments, and the lymphatic vessels were traced for a considerable part of their course. They travelled from the caudal to the cephalic surface of the diaphragm, but their cavities were always separated from the cavity of the peritoneum by two layers of flat cells separated by a basement membrane. An attempt was made to stain the connecting tissue between the endothelial cells of the peritoneum by means of silver nitrate. Although the intercellular tissue bounding the endothelium was visualized, no evidence of the presence of stomata was discovered. These observations confirm those of Cunningham, which have been mentioned above. In the search through the serial sections of these diaphragms a very large number of endothelial cells and leucocytes passed under review. In no instance was an endothelial cell or a leucocyte seen to contain an avian cell. If the transportation of these foreign avian cells from the peritoneum to the peripheral circulation takes place by a process of phagocytosis, it must be more than coincidence that no evidence of phagocytosis was found in the present series, more especially as in most of the mice large numbers of avian cells were seen in the peripheral circulation and the diaphragmatic lymphatics, and also many of the lymph-glands in the mediastinum were found to contain quantities of them. The spleens of the mice in Table III were weighed with a view to ascertaining the destination and fate of the transfused cells, but no definite conclusions have been reached on this point.

SUMMARY AND CONCLUSIONS.

(1) Guinea-pigs, rabbits, mice and a boy were given intra-peritoneal injections of autogenous and heterogeneous blood. This was at first citrated; in some of the later experiments it was defibrinated. The absorption of blood from the peritoneal cavity was observed, and the transfused cells sought for in the peripheral circulation of the recipients where their life period was followed. Histological examinations were made, and serial sections cut, from the diaphragms and the spleens of some of the mice.

(2) A compatible blood injected into the peritoneal cavity causes no untoward symptoms in animals. The peritoneum does not appear to be adversely affected by the presence of blood within it.

(3) Citrated blood clots.

(4) Blood is absorbed rapidly from this peritoneal clot, and the cells can be found in the peripheral circulation. It acts, therefore, as a slowly given blood transfusion.

(5) Blood-cells that remain unabsorbed in the peritoneal cavity after 48 hours are found to have retained their structure.

(6) The transfused cells enter the lymphatics, and can be seen in large quantities in the diaphragmatic lymphatic vessels within 1 hour of the transfusion.

(7) Avian cells can live in the circulation of mice for a period up to 56 hours. Their numbers are greatest in the peripheral circulation at the end of 24 hours, after which they gradually disappear. No degenerate cells were seen in the peripheral circulation, although in the experiments in which guinea-pigs were used a few mononuclears were seen containing phagocytosed hen's blood-cells.

(8) Reviewing the above experiments I am inclined to believe that the immigration of cells from the peritoneum is a mechanical process, but as to the exact mechanism I am still in doubt.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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THE INFLUENCE OF SERA OBTAINED FROM CASES OF STREPTOCOCCAL SEPTICÆMIA ON THE VIRULENCE OF THE HOMOLOGOUS COCCI.*

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Received for publication September 1st, 1927.

It has been shown in a previous paper (1927[2]) that the degree of virulence of hæmolytic streptococci for mice can, to some extent, be estimated *in vitro* by determining the rate of multiplication of the cocci in fresh defibrinated human blood. Hæmolytic streptococci which have been recently isolated by blood-culture from cases of septicæmia and which are highly virulent to mice multiply rapidly in human blood, but when the same strain has become attenuated by cultivation on agar, so that a very large dose is required to kill a mouse, the cocci are no longer able to multiply in human blood. The rate of multiplication of hæmolytic streptococci in fresh defibrinated human blood can therefore be used as an index of virulence.

* The expense of this research was partly defrayed by a grant from the Medical Research Council.

It has also been shown (1927[3]) that the filterable toxin of hæmolytic streptococci, recognizable by the Dick test, is toxic to human blood, and that this toxic action can be neutralized by antitoxin. In the present communication another effect of serum from cases of septicæmia is recorded which appears to be distinct from antitoxic action, and to depend on an antibody contained in the serum capable of attenuating the virulence of the infecting cocci. It is frequently observed that hæmolytic streptococci freshly isolated from the blood-stream have not attained their maximum level of virulence, as measured by their rate of multiplication in normal human blood, but can be further exalted by cultivation in normal human serum *in vitro*. This is a possible indication that there is some influence in the body capable of checking the virulence of the infecting cocci, and further evidence of a similar kind is obtained from experiments on mice. If virulent hæmolytic streptococci are inoculated into the peritoneal cavities of a number of mice, and if the virulence of the cocci subsequently recovered from the heart's blood of the mice is tested by determining their rate of multiplication in normal human blood, it will be found that in most cases the cocci have acquired an increased rate of multiplication in blood, but in some cases it will be observed that the multiplication rate of the cocci has been diminished, possibly owing to the elaboration of an antibody by the mice capable of attenuating the virulence of the infecting cocci.

The following experiments show that serum from a case of puerperal septicæmia was able to attenuate the virulence of the homologous cocci *in vitro*.

Characteristics of the strain of streptococcus and of the case from which it was isolated.—The strain of hæmolytic streptococcus used in these experiments was isolated by blood-culture from a case of puerperal septicæmia. It was examined by Eagles (1926) for its power of producing a filterable toxin which would cause a positive Dick reaction, and its agglutination reactions were also examined by him. He found that filtrates prepared from this strain did not produce a positive Dick reaction when injected intradermally in a dilution of 1 in 10. I have also prepared a number of filtrates from this strain, and have tested their toxicity on human blood *in vitro* with completely negative results. It is therefore possible to exclude from the following experiments the effects of filterable toxin, and presumably the effects of antitoxin can be excluded on the same grounds.

At the time when the serum was obtained from the patient she had a positive blood-culture with about 1000 hæmolytic streptococci per c.c. of her blood. The serum was sterilized before use by heating for one hour at 56° C.

Technique.—In all these experiments virulence was estimated by determining the power of the cocci to multiply in human blood, and owing to the small amount of the patient's serum available it was impossible to carry out any experiments on its protective properties for mice.

The technique, which has been previously described (1927[1]), was as follows:

(1) 5 c.mm. of the patient's serum was placed in a glass capsule and a control capsule was prepared containing the same volume of normal human serum.

(2) 15 c.mm. of a broth culture of the patient's streptococcus, suitably diluted with normal saline, was added to 300 c.mm. of defibrinated normal human blood, and, after thorough mixing, the number of streptococci planted in the blood was determined by plating out a volume of the blood in agar.

(3) 100 c.mm. of the infected blood was transferred to each of the capsules, which were immediately sealed and, incubated on a mixing machine. After 3 hours' incubation equal volumes of blood from the two capsules were plated out in agar, and after incubating the plates for 36 hours the colonies were counted. When highly virulent cultures were used it was necessary, owing to rapid multiplication of the cocci, to dilute the blood before plating in order to obtain a countable number of colonies.

THE ACTION OF THE PATIENT'S SERUM ON THE HOMOLOGOUS STREPTOCOCCI IN NORMAL HUMAN BLOOD.

The following experiment shows the effect of the patient's serum on a highly virulent culture of her own streptococcus:

Number of colonies obtained from 50 c.mm. of blood before incubation		7,800
,, ,,	obtained from 50 c.mm. of blood + normal serum 1 in 20 after incubation	44,000
,, ,,	obtained from 50 c.mm. of blood + patient's serum 1 in 20 after incubation	129

The virulent streptococci multiplied freely in the blood to which normal serum had been added, but they were unable to multiply in the blood containing the patient's serum. This serum not only prevented multiplication, but it caused a great reduction in the number of cocci in the blood.

It will be shown later that this phenomenon is not due to agglutination of cocci producing a smaller number of colonies, or to any bactericidal power possessed by the patient's serum, and evidence will be brought forward to show that it is due to an attenuating influence of the patient's serum on virulent cocci.

It has been shown in a previous paper (1927[2]) that virulent freshly isolated hæmolytic streptococci become attenuated when the cocci are cultivated on agar or in broth, and that, under certain conditions, the lost virulence can be restored by cultivation in normal human serum. The attenuating action of the patient's serum was tested at intervals with cocci which were subcultured daily in broth, and it was found that the effect of the serum diminished *pari passu* with the attenuation of the cultures. In other words, as the cocci became less virulent, the attenuating action of the serum became less apparent, but when the lost virulence of the cocci was subsequently restored by cultivation in normal serum, it was found that the patient's serum still possessed its original attenuating power.

TABLE I.—*The Action of the Patient's Serum on Virulent and Attenuated Cultures of her own Streptococcus.*

	Number of cocci planted in 50 c.mm. of blood.	Number of colonies cultivated from 50 c.mm. of the following mixtures after 3 hours' incubation on the mixing machine.		Dates of experiments.
		Infected blood + control serum 1 in 20.	Infected blood + patient's serum 1 in 20.	
Freshly isolated culture	4,000	298	7	3.7.25
Culture attenuated by cultivation in broth	156,000	864	377	2.9.25
Culture with virulence restored by cultivation in normal human serum	1,300	384	10	5.11.25

The patient's serum attenuated the freshly-isolated culture to such an extent that normal blood + patient's serum was able to kill 40 times more cocci than the control, but when the culture had been attenuated by cultivation in broth the ratio was reduced from 1/40 to 1/2½. Subsequently the lost virulence was restored by cultivation in normal human serum, and with this culture normal blood + patient's serum was able to kill 38 times more cocci than the control.

It appears that the action of the patient's serum is dependent on the virulence of the culture used in the test. If an attenuated culture is used the serum is given a smaller range of action, as it cannot attenuate cocci which have already been attenuated by other means.

AN ALTERNATIVE METHOD OF SHOWING THE ATTENUATING ACTION OF THE PATIENT'S SERUM.

If a small number of streptococci from a moderately-virulent culture are exposed to the action of the patient's serum for 3 hours at 37°C., and if a broth culture, from the cocci so treated, is subsequently tested for virulence by determining its power of multiplication in human blood, it will be found that the virulence of the culture has been attenuated by the patient's serum. There are, however, several difficulties to be overcome before the action of the patient's serum can be demonstrated in this way. If a large number of cocci are added to the serum, its attenuating influence is not apparent. If a small number of cocci are added to the serum they multiply freely during the 3 hours' incubation, so that the conditions at the end of the experiment are similar to those which occur when a large number of cocci are added to the serum. It is therefore necessary to restrain the multiplication of the streptococci during the time when they are exposed to the attenuating influence of the serum. This can be done by diluting the serum with normal saline. If a small number of streptococci are added to normal saline and incubated at 37° C. for 3 hours they all die and the saline becomes sterile. By adding a small quantity of serum to the saline complete sterilization is avoided and

multiplication of the cocci is prevented. This method was therefore used to demonstrate the attenuating action of the patient's serum.

Two capsules were prepared, one containing 5 c.mm. of normal serum and the other 5 c.mm. of the patient's serum. Normal saline 300 c.mm. was infected with 15 c.mm. of a culture of moderate virulence diluted 1 in 2000 in normal saline. Each of the capsules received 100 c.mm. of the infected saline and they were then incubated on the mixing machine for 3 hours. After incubation two broth cultures were prepared by transferring 5 c.mm. of fluid from each capsule to 10 c.c. of broth. In order to exclude the effect of the small volume of patient's serum carried over with the infecting streptococci to the broth culture, an additional control broth culture was prepared as follows: A capsule containing 5 c.mm. of the patient's serum and 100 c.mm. of uninfected normal saline was incubated on the mixing machine with the other capsules. After incubation 5 c.mm. of fluid was transferred from this capsule to a tube containing 10 c.c. of broth, which was then inoculated with 5 c.mm. of fluid from the capsule which contained normal serum and infected saline. By this means a control culture was prepared which contained patient's serum and cocci which had not been previously exposed to the action of this serum. The ultimate dilution of serum in the broth cultures was 1 in 42,420. Before testing the virulence of the broth cultures on normal human blood quantitative counts were made in blood-agar to make sure that there had been no multiplication of cocci during their incubation in dilute serum.

The broth culture derived from cocci which had been exposed to the action of the patient's serum was less virulent to normal human blood than either of the other cultures.

This is not an easy experiment to carry through successfully, as multiplication of the cocci during exposure to the attenuating action of the serum must be avoided and small implants must be used in the initial process. If a comparatively large number of cocci are added to the salt solution at the beginning of the experiment, the result will be much less striking, and may be missed altogether.

AN EXAMINATION OF SOME OTHER CHARACTERISTICS OF THE PATIENT'S SERUM.

Reference has already been made to the possibility that agglutination might reduce the number of colonies which could be cultivated from blood + patient's serum. To investigate this possibility the original experiment, which showed the attenuating action of the patient's serum in normal human blood, was repeated, but the original experimental conditions were altered by substituting normal saline or normal serum for blood, and it was found that in the absence of blood-cells the patient's serum caused no reduction in the number of colonies which could be cultivated from the mixtures. In these experiments the only variation from the technique previously described was the substitution of infected saline or serum for infected blood, and they therefore show that the phenomenon was not due in any degree to agglutination or to any direct bactericidal power possessed by the patient's serum.

The possibility that the phenomenon was in part due to opsonization of

TABLE II.—*Bactericidal Action of Normal Blood on a Broth Culture derived from Streptococci which had been Exposed to the Attenuating Influence of the Patient's Serum and on two Control Cultures.*

Dilution of cultures.	Control: Broth culture derived from saline and normal serum.		Broth culture derived from saline and patient's serum.		Control: Broth culture derived from saline and normal serum (patient's serum added to broth).	
	Number of cocci planted in 50 c.mm. of blood.*	Number of colonies cultivated from 50 c.mm. of blood after 3 hours' incubation on mixing machine.	Number of cocci planted in 50 c.mm. of blood.*	Number of colonies cultivated from 50 c.mm. of blood after 3 hours' incubation on mixing machine.	Number of cocci planted in 50 c.mm. of blood.*	Number of colonies cultivated from 50 c.mm. of blood after 3 hours' incubation on mixing machine.
1 in 50	19,000	+++	30,800	715	28,600	+++
1 in 500	1,900	297	3,080	16	2,860	288
1 in 5,000	190	14	308	0	286	18
1 in 15,000	63	4	103	0	95	10

* The figures in the upper part of this column were calculated from a count of the implants used in the lower dilutions.

the streptococci leading to increased phagocytosis was also investigated. The opsonic technique was used to investigate the influence of the patient's serum on phagocytosis by comparing the number of streptococci ingested by leucocytes from a mixture of normal blood + patient's serum (1 in 20) with the number ingested by leucocytes from a control mixture of normal blood + normal serum (1 in 20). Two parts of each of these mixtures were incubated for 15 minutes with one part of an emulsion of the patient's streptococcus, and opsonic counts showed that under these conditions the patient's serum did not cause an increase in phagocytosis. It has been shown in a previous paper (1927[2]) that the characteristics of virulent cultures of hæmolytic streptococci are resistance to phagocytosis and a high rate of multiplication in normal human serum—two separate qualities which together enable virulent cocci to multiply rapidly in normal human blood. In the phagocytic experiment quoted above, 15 minutes' incubation was not long enough to cause attenuation of cocci by the patient's serum—a change which would have allowed the leucocytes to ingest a larger number of cocci, but it was sufficient to show any increased opsonization which might have occurred.

It has already been stated that the patient's streptococcus did not produce a filterable toxin, so that the action of her serum cannot be attributed to neutralization of such a toxin. It is therefore probable that the effect of the patient's serum on the bactericidal action of normal blood was largely, if not entirely, due to attenuation of virulent cocci.

TABLE III.—*The Influence of Sera from Cases of Septicæmia on the Bactericidal Power of Normal Blood for the Homologous Cocci.*

Identification numbers of sera.	Infecting organism.	Number of cocci planted in 50 c.mm. of blood.	Number of colonies cultivated from 50 c.mm. of the following mixtures after 3 hours' incubation on the mixing machine.	
			Infected blood + control serum 1 in 20.	Infected blood + patient's serum 1 in 20.
2	Hæmolytic streptococci	2,560	332	223
3	Ditto	12,400	1,004	230
4	,,	206	259	3
5	,,	1,750	631	255
6	,,	385	226	136
7	,,	—	237	65
8	*Streptococcus viridans*	1,500	33	34
9	Ditto	2,200	11	14
10	*Staphylococcus aureus*	500	90	145
11	Ditto	3,400	62	154
12	*Micrococcus tetragenus*	1,000	664	676

INVESTIGATION OF SERA FROM OTHER CASES OF SEPTICÆMIA.

A number of sera, obtained from cases of septicæmia infected with various organisms, have been tested for their power of increasing the bactericidal action of normal blood on a recently-isolated culture of the homologous cocci. Table III shows that all the sera from patients infected with hæmolytic streptococci were able to increase the bactericidal action of normal blood on the homologous cocci, but that in some cases the effect of the patient's serum was very small. No steps were taken in these experiments to exclude the effect of antitoxin in the patient's serum, and as some of the strains were highly toxigenic, it is probable that neutralization of filterable toxin had a considerable influence in these experiments. The sera derived from cases of septicæmia caused by organisms other than hæmolytic streptococci were each tested in the same way, but they did not increase the bactericidal action of normal blood on the homologous cocci, and the sera from staphylococcal septicæmias had a slight toxic action on normal blood.

SUMMARY.

Virulent hæmolytic streptococci, isolated by blood-culture from a case of puerperal septicæmia, multiplied rapidly in normal human blood, but when a small quantity of the patient's serum was added to the normal blood multiplication was prevented, and the blood had a considerable bactericidal action on the cocci.

Experiments are quoted to show that this phenomenon is not caused by (i) a direct bactericidal action of the serum on the cocci; (ii) agglutination; (iii) increased opsonization; (iv) neutralization of filterable toxin; (v) action of the serum on normal leucocytes.

The cocci are in some unknown way altered by the serum so that they are more easily killed by blood, and it is probable that the phenomenon is due to an attenuating action of the serum on the virulence of the cocci, because (i) the serum acts powerfully on virulent cocci but only slightly on attenuated cocci; (ii) attenuated broth cultures can be prepared from virulent broth cultures by exposing the cocci to the action of the serum.

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ON THE ELECTRICAL BEHAVIOUR OF THE BACTERIOPHAGE.

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Received for publication August 17th, 1927.

THE following experiments on the bacteriophage for *B. shiga* arose incidentally in the course of inquiries into the electrical behaviour of certain of the pathogenic viruses and the search for a satisfactory apparatus for observing their migration in an electric field. The different forms of apparatus tried were at first tested with various colloidal dyes, but it was thought desirable to use some indicator more closely resembling the viruses, and the Shiga bacteriophage was selected as probably representing particles of the same order of magnitude as the pathogenic viruses under investigation, and as having the advantage of not necessitating experiments on animals.

A review of the literature, however, showed that observers are by no means in agreement as to the sign of the charge carried by the phage, so that it became necessary in the first instance to settle this point.

It is somewhat remarkable that so little precise information is available concerning the electric charges carried by the particles of the ultramicroscopic viruses—particularly in view of the large amount of attention which has been directed towards the question of their passage through different types of filter. In the filtration of colloidal solutions or fine emulsions through the various forms of filter commonly in use—such as the Berkefeld, Chamberland, etc.—the process was originally regarded as being purely mechanical, the filter, with its fine capillary channels, being supposed to act like a sieve in preventing the passage of particles whose diameter was greater than that of the pores of the filter. It is now, however, generally recognized that an important factor in the process is the adsorption of the colloid or suspended matter on the walls of the capillary channels of the filter, so that under suitable conditions a filter may be capable of stopping the passage of particles whose diameter is markedly less than that of the pores of the filter used. The chief factor in this adsorption is believed to be the relations existing between the electric charges on the particles of the colloid or suspension and those of the material of the filter.

The *rôle* of electrical adsorption was first demonstrated in connection with dying and staining by Bayliss (1906) in his experiments on the taking up of Congo-red by filter-paper, and has later been studied in the case of filtration through Berkefeld and similar filters by a number of workers, notably by Mudd (1922–3), who states the case very clearly as follows: "The contact

surfaces of the pores of a Berkefeld filter and the fluid bathing them is the site of an electrical potential difference, an ordinary Helmholtz double layer, the solid walls carrying a negative and the liquid a positive charge. We should predict, therefore, that when solutions or suspensions are filtered, positively charged particles, at least to some extent, would be absorbed on the filter walls and thus removed from solution, while negatively charged particles would be borne through by the filtration stream. This expectation we find fulfilled with dyes. Solutions of " basic " dyes like methylene blue in which the coloured ion is electro-positive may be completely decolorized by the filter at first, only the latter portions of the filtrates being coloured by the dye. Solutions of " acid " dyes like eosin, in which the coloured ion is negative, pass through a Berkefeld candle without demonstrable change."

These facts have an obvious practical bearing on the question of filtration in general, but more particularly on the filtration of the so-called ultra-microscopic or filter-passing viruses, as estimates of the size of the particles of a particular virus have been largely based on the size of the pores of the finest filter through which the virus will pass, commonly without reference to the sign and magnitude of the respective electric charges on the particles and on the filter, although these charges may be important, or perhaps dominating, factors in the process.

Opinions on the sign of the electric charge carried by the bacteriophage have been mainly based on the behaviour of the phage towards various electro-positive and electro-negative powders and colloids, and different observers are by no means in agreement as to whether the phage must be regarded as carrying a positive or a negative charge.

d'Herelle (1926) states that the " corpuscles " of the bacteriophage possess a negative charge, and that the bacteriophage is thus a negative colloid. He does not appear to have actually tested the movement of the phage in an electric field, but deduces his opinion from the observation that the phage is not adsorbed by the substance of Chamberland or Berkefeld candles, does not react with negative colloids, and is flocculated by acids, but not by alkalies. He points out, however, that certain races of the phage can act in a frankly acid medium (pH = 5·1), and suggests that possibly these differences in properties may be associated with quantitative differences in the electric charge.

Seiffert (1923–4), on the other hand, found that the phage was practically entirely removed by shaking with kieselguhr, but not by shaking with bolus alba, and so concludes that the phage has a positive charge. The reaction at which the process was carried out is not stated.

Prausnitz and Firle (1924) regard the phage as having an electro-positive charge, basing this view on the adsorption of the phage by electro-negative colloids.

Gildemeister and Hertzberg (1924) found that the phage is adsorbed by kieselguhr in acid solutions, but can be recovered by the addition of ammonia. Also that filters can be made impermeable to the phage by increasing the hydrogen-ion concentration ; whilst phages which pass the filter with difficulty can be made to pass easily by increasing the OH-ion concentration.

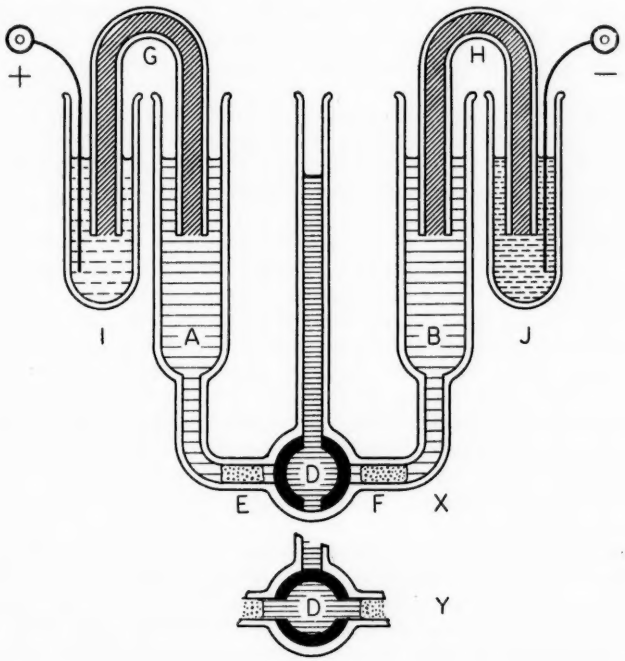
Eliava and Suarez (1927), on the strength of their experiments on the

passage of the phage through collodion membranes, agree with d'Herelle in believing that its charge is negative.

The only record of actual tests of the migration of the phage in an electric field which the writer has been able to find in the literature is the work of K. Koch (1926), who tested the cataphoresis of a Shiga bacteriophage, and in two experiments, working with an E.M.F. of 200 volts, found a migration towards the cathode, showing that the phage had a positive charge. These experiments are not very convincing, and no statement is made of the reaction of the broth in which the migration was observed.

CATAPHORESIS EXPERIMENTS.

After experiments with several different forms of cataphoresis apparatus, the one figured in the half-scale diagram below was finally adopted.



This consists of a central tap (D) with a hollow barrel which can be turned so as either (*a*) to make communication with the central limb (C) only, or (*b*) to connect with one another the two side limbs (A) and (B) which constitute the positive and negative limbs of the apparatus.

The electric current is supplied to these limbs through glass tubes, filled with 1 % sodium chloride agar gel, dipping at their distal ends into the tubes

(I) and (J) which contain the non-polarizable electrodes, the positive electrode being a strip of silver foil immersed in a solution of sodium chloride, and the negative electrode a copper wire in a solution of bichloride of copper containing at the bottom some crystals of the salt. The copper wire is insulated with a coating of paraffin wax, except at its lower extremity, where it is in contact with a saturated solution of the salt.

Loose plugs of absorbent cotton-wool are placed in the horizontal limbs of the apparatus at (E) and (F) so as to minimize the formation of currents.

In carrying out an experiment, the three limbs of the apparatus were filled to a convenient height with the saline or buffer solution in which the migration of the phage was to be observed. The two agar-filled connecting tubes were then placed in position, and the tap turned so as to connect the positive and negative limbs, and equalize the levels on the two sides. This having been done the tap was turned through an angle of 90°, and approximately half a cubic centimetre of the filtrate containing the bacteriophage was introduced by means of a pipette into the hollow barrel of the tap, which had a capacity of about 4 cubic centimetres, and thorough mixing ensured by drawing the mixture into the pipette and blowing it out several times.

The apparatus was allowed to stand for a sufficient time to eliminate any temperature differences, and then the tap turned back through an angle of 90° so as to bring the positive and negative limbs into connection with one another through the central chamber in the barrel of the tap. The electric current was then switched on.

The E.M.F. used was 200 volts, with a current of from $1\frac{1}{2}$ to $6\frac{1}{2}$ milliampères. A milliampère-meter was included in the circuit together with a variable resistance and also a lamp resistance as a matter of precaution.

After the current had passed for varying periods, the connection between the two limbs was interrupted by turning the tap, and samples of the fluid in each limb were taken with fine capillary pipettes and tested for the presence or absence of the phage. These samples were taken from the junction of the wide and the narrow portions of the vertical limbs, and in order not to interfere with the relative levels, the same number of drops (commonly three) were taken from each limb, any surplus fluid being returned to the limb from which it was taken. After taking the samples, the tap was again opened and the experiment continued.

The presence or absence of the phage was determined by adding the three drops of fluid to a tube of nutrient broth, which, together with a control tube of broth only, was then inoculated with a loop of *B. shiga* and placed in the incubator. The tubes were examined on the following day, when any tubes containing the phage were conspicuous by the complete absence of growth.

The apparatus as described above was found to be very practical, its simplicity rendering it easy to manipulate and to clean. It is, moreover, easily sterilized by boiling. The absence of direct communication with the central limb during the course of a test avoids the risk of any flow from this limb on the removal of the samples, and the agar-filled tubes are of such a length as to prevent the entry of electrolytic products from the electrodes into the apparatus. This was verified after each experiment, the reaction of the fluid

in the two limbs being tested by the addition of a suitable indicator to ensure that no change in reaction had taken place.

As the distance from the central chamber to the sampling points is relatively short, results are obtained quickly, and no difficulty has been met with from the presence of convection currents.

The particular strain of bacteriophage used for these experiments was isolated from the droppings of normal fowls, and its activity was increased by cultivation in broth with *B. shiga* and subsequent filtration. This process was repeated five times. The final filtrate was kept in small sealed tubes at room temperature, and at the time of the experiments was found to be active at 1/10,000,000 c.c., *i.e.* the addition of this amount to a tube of broth inoculated with *B. shiga* prevented the appearance of any visible growth after 24 hours in the incubator.

No attempt was made to purify the phage by dilution or plating.

After a preliminary trial with normal saline all the cataphoresis tests were made with buffer mixtures of known reaction.

In the first experiments solutions were used which were practically neutral, and as in these the phage showed a definite migration towards the anode—indicating that, in the neighbourhood of neutrality, it carries a negative charge—a series of tests was made at increasing degrees of acidity in order to ascertain whether the sign of the charge is altered at higher hydrogen-ion concentrations.

The table on p. 374 shows the results obtained in the experiments made.

The reactions given in the table are the reactions of the fluid with which the apparatus was filled. In the central chamber of the tap the reaction is rather less acid owing to the addition of the alkaline filtrate containing the phage, but this is only material in the case of tests carried out at comparatively high acidities, and even then does not involve a great change in the reaction: thus, in the experiment made at the highest degree of acidity (pH = 3·2), the fluid in the barrel of the tap after the addition of the filtrate was found to be pH = 3·6.

It will be seen that in thirteen out of the fifteen experiments made the phage showed a migration towards the anode, while in the remaining two no migration in either direction was observed. In no case was there migration towards the cathode, although the acidity was increased as far as was possible without destroying the phage.

Thus over the range from pH = 3·6 to pH = 7·6 the phage shows a definite migration towards the anode, so that over this range of reaction it must be regarded as carrying a negative charge.

It was suggestive that the two tests which showed no migration both occurred near the acid end of the series, and it appeared not unlikely that these negative results were due to the fact that sufficient time had not been allowed for the migration, as if the increased acidity had diminished the negative charge on the phage, migration would take place more slowly; moreover, there might be some flocculation of the phage, as suggested by Da Costa Cruz (1924), who was of the opinion that the phage is flocculated in acid media.

If the amount of the negative charge on the phage is lowered by the

increased hydrogen-ion concentration, it seems probable that this would show itself in a diminished ability of the phage to pass through a filter of porous earth, which itself has a negative charge; and any flocculation of the phage consequent on the increased acidity would naturally affect the process in the same sense.

pH.	Medium in which migration was tested.	Current in milliampères.	Time of sampling.	Presence or absence of phage in— Anode limb.	Kathode limb.
3·2	M/5 Acetate acetic acid	2	2 hours	+	0
			4½ „	+	0
3·5	M/5 Acetate acetic acid	3	2 „	0	0
4·0	M/50 Acetate acetic acid	1½	2½ „	+	0
4·5	M/50 Acetate acetic acid saline	6	1¼ „	0	0
5·0	M/50 Acetate acetic acid	3½	1 hour	+	0
			2 hours	+	0
5·0	M/50 Citrate hydrochloric acid	...	1 hour	+	0
			2 hours	+	0
			3 „	+	0
5.25	M/50 Acetate acetic acid	3½	1 hour	+	0
			2 hours	+	0
5·5	M/100 Citrate hydrochloric acid	5	1 hour	+	0
			2 hours	+	0
			3 „	+	0
5·5	M/50 Acetate acetic acid	3½	1 hour	+	0
			2 hours	+	0
6·0	M/100 Phosphate	4½	1 hour	+	0
			2 hours	+	0
			3 „	+	0
6·0	M/50 Acetate acetic acid	3½	1 hour	+	0
7·4	M/100 Phosphate saline	...	5 hours*	+	0
7·4	M/100 Phosphate saline	6½	1 hour	+	0
			2 hours	+	0
			3 „	+	0
7·6	M/100 Phosphate saline	...	4¾ „ *	+	0
7·7	Unbuffered saline	...	5 „ *	+	0

* In these three experiments an older type of apparatus was used.

That this actually is the case was shown by Gildemeister and Hertzberg (1924), as mentioned above.

In order to verify this point and to observe the degree of acidity at which the change in the permeability of the filter for the phage begins, a short series of separate filtration tests was carried out on dilutions of the phage in buffer mixtures at various reactions.

Filtration Tests.

In the first instance two dilutions of 1% of the original filtrate containing the phage were made up in M/50 acetate-acetic acid buffer solutions of pH =

4·5 and pH = 6·0 respectively, and at once separately filtered through new small Mandler candles at the same rate (approximately 2 c.c. per minute), about 30 c.c. being filtered in each case. The two filtrates were tested by the addition of varying quantities of the filtrates—in multiples of ten—to tubes of broth, which were then inoculated with *B. Shiga* and incubated over-night. Next morning any tubes containing the phage were noted by the absence of growth.

Control tests were made in the same way with the unfiltered dilutions.

In order to ascertain if the two candles used were of approximately the same degree of porosity, these after use were washed and sterilized, and on the following day the test was repeated in exactly the same manner, except that the candle which had been used on the first occasion for the more acid solution was used next day for the more alkaline, and *vice versâ*. As the results obtained were identical in the case of the two candles, it was assumed that—under the conditions of the experiment—these candles could be regarded as of approximately the same degree of porosity, and they were used in a series of tests which were made with dilutions of the phage in buffer mixtures of different reactions.

The results of these tests are summarized in the following table:

Table showing the Influence of the Hydrogen-ion Concentration on the Filtration of the Bacteriophage.

Volume of filtrate added to broth tube.	Reaction at which the filtration was carried out.					
	3·5.	4·0.	4·5.	5·0.	5·5.	6·0.
After filtration.						
1·0 c.c.	0	0	0	0	0	0
0·1 c.c.	+	0	0	0	0	0
0·01 c.c.	+	+	0	0	0	0
0·001 c.c.	+	+	+	0	0	0
0·0001 c.c.	+	+	+	0	0	0
0·00001 c.c.	+	+	+	0	0	0
0·000001 c.c.	+	+	+	+	+	+
0·0000001 c.c.	+	+	+	+	+	+
Unfiltered control.						
0·0001 c.c.	+	0	0	0	0	0
0·00001 c.c.	+	0	0	0	0	0
0·000001 c.c.	+	+	+	+	+	+
0·0000001 c.c.	+	+	+	+	+	+
0·00000001 c.c.	+	+	+	+	+	+

\+ represents turbidity from the growth of *B. Shiga*.
0 represents absence of visible growth.

The results of these tests are very striking as showing the effect of the reaction on the character of the filtration. In the neighbourhood of neutrality the phage passes through the Mandler candle practically without hindrance. This continues to be the case as the acidity is increased until a reaction of

pH = 5·0 is reached, when a sudden change takes place; at pH = 4·5 it is found that the amount of the phage passing the filter is only one thousandth part of the amount passing at pH = 5·0. At pH = 4·0 this amount is reduced to one ten thousandth.

The tests cannot usefully be carried further, as at pH = 3·5 the control tests on the unfiltered dilutions show that the phage is beginning to suffer from the excessive acidity.

SUMMARY.

1. A simple and practical form of cataphoresis apparatus is described.

2. Investigation of the electrical migration of a bacteriophage for *B. Shiga* at a number of points over a range of reactions between pH=3·6 and pH=7·6 show that between these points the phage regularly migrates towards the anode, showing that over this range it has a negative charge.

3. Filtration tests emphasize the effect of the reaction on the process of filtration. In the neighbourhood of neutrality the phage passes through a Mandler candle practically without hindrance. This continues to be the case until a reaction of pH = 5·0 is reached, when a sudden change takes place, and at pH = 4·5 the amount of phage passing the filter is only one thousandth part of the amount passing at pH = 5·0. At pH = 4·0 this amount is reduced to one ten thousandth.

The author's thanks are due to Dr. E. H. J. Schuster for the diagram of the cataphoresis apparatus used in these experiments.

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TETANUS.—V. THE LOCAL FATE OF TETANUS SPORES INOCULATED INTO GUINEA-PIGS.

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Received for publication July 26th, 1927.

THE following experiments were carried out with the advice of Dr. Paul Fildes in an endeavour to study the local histological changes which follow the inoculation of toxic and non-toxic tetanus spores into the tissues of the guinea-pig. In particular attention was directed to the circumstances in which vegetation of these spores occurs.

Note on Staining Methods Used.

In the absence of any prescribed method for staining tetanus spores in paraffin sections of tissues, a preliminary investigation was carried out in which it was found that spores can be satisfactorily demonstrated by the following modification of the carbol-fuchsin and methylene blue method commonly used for films:

1. Remove paraffin by xylol, and xylol by absolute alcohol.
2. Cover slide with boiling carbol-fuchsin and leave 30 seconds.
3. Wash in tap-water.
4. Differentiate in acid alcohol till section is pale red.
5. Wash in tap-water.
6. Counterstain with Loeffler's methylene blue for 1 minute.
7. Wash in tap-water.
8. Dip rapidly into acid alcohol and wash again.
9. Dehydrate, clear, and mount in Canada balsam.

The spores are bright red, nuclei blue and vegetating bacilli dark blue. The differentiation is sharp. The cytoplasm, however, is often so decolourized that it is difficult to be certain whether the spores lie within a cell or without. This point can be determined by staining sections in the methylene blue for 10 seconds only and omitting the subsequent differentiation in acid alcohol; by this modification the cytoplasm is stained brightly, but the nuclei are overstained and less sharply differentiated.

Spores can also be demonstrated in paraffin sections by a combination of carbol fuchsin and Ehrlich's hæmatoxylin. Schmorl's (1905) modification of the Ziehl-Neelsen method is very satisfactory.

* Working under grants from the Medical Research Council.

I. SUBCUTANEOUS INOCULATIONS.

The fate of toxic and non-toxic spores, either alone or with the addition of earth, was traced in a series of subcutaneous inoculations.

Experiment 1.—Four batches of 6 guinea-pigs were inoculated subcutaneously on the outer side of the right thigh as follows:

Batch I: 0·2 c.c. suspension of non-toxic spores.

Batch II: 0·1 c.c. suspension of non-toxic spores + 0·1 c.c. sterile suspension of earth.

Batch III: 0·2 c.c. suspension of toxic spores.

Batch IV: 0·1 c.c. suspension of toxic spores + 0·1 c.c. sterile suspension of earth.

The spores used were obtained from the toxic strain TS 40 and its non-toxic variant separated by Fildes (1927). The strength of the suspension was fifteen million spores per cubic centimetre.

The earth used had been brought from Amesbury, in Wiltshire, a district in which there is a chalk substratum. The suspension was prepared by grinding up and suspending 10% of earth in distilled water for 5 minutes. The supernatant turbid fluid was then decanted and autoclaved.

One guinea-pig in each batch was subsequently killed after 4 hours, and 1, 2, 3, 4 and 5 days.

The skin and subcutaneous tissues were divided vertically through the site of inoculation. One half of the lesion was removed and fixed in 2% saline formaldehyde, the other was used in preparing films.

The experiment was a failure inasmuch as general tetanus did not develop in any instance, nor was there any evidence of vegetation in either films or sections. The procedure was therefore repeated using a heavier dose of earth.

Experiment 2.—The suspension of earth was prepared as before, but 1 minute only was allowed for sedimentation.

20 guinea-pigs were divided into four batches and inoculated beneath the skin of the abdominal wall as follows:

Batch I: 0·2 c.c. suspension of non-toxic spores.

Batch II: 0·2 c.c. suspension of non-toxic spores + 0·2 c.c. sterile suspension of earth.

Batch III: 0·2 c.c. suspension of toxic spores.

Batch IV: 0·2 c.c. suspension of toxic spores + 0·2 c.c. sterile suspension of earth.

The animals were subsequently killed at intervals of 1, 2, 3, 4 and 6 days. On the morning of the 4th day one of the two remaining guinea-pigs in Batch IV was found dead. The last guinea-pig of this batch showed slight local tetanus on the 4th day and was found dead on the morning of the 5th day. All the guinea-pigs in the other batches were healthy up to the time of death.

Macroscopic Appearances of Lesions.

The lesions produced by spores alone (Batches I and III) differed in appearance from those produced by a mixture of spores and earth (Batches II and IV).

When spores alone were inoculated the result in 24 hours was a moderately well defined firm opaque white mass of the shape and about the size of a lentil. There was considerable œdema of the surrounding soft tissues. After 48 hours and subsequently the mass slowly decreased in size and became tougher in consistency. The œdema also diminished.

When a mixture of spores and earth was inoculated the lesion produced appeared to be from four to six times as large as that produced by spores alone. It consisted of a sharply defined abscess, with greenish-grey diffluent contents, which bulged up beneath the skin of the abdominal wall. There was great œdema of the neighbouring soft tissues, which subsided gradually after the first 24 hours. There was no appreciable alteration in the size of the abscess during the first 6 days. The centre remained soft and diffluent.

Microscopic Appearances of the Lesions.

(a) *Spores alone inoculated.*—In sections of the skin and subcutaneous tissue taken 4 hours after inoculation (Exp. 1) a considerable collection of round cells is seen at the site of inoculation. In this collection polymorphonuclear leucocytes predominate, but amongst them and especially in the periphery there are a few scattered large mononuclear cells. Phagocytosis of the spores is, at this stage, exhibited only by the polymorphonuclear leucocytes. About 80% of the spores appear to have been taken up. A few faintly stained bacillary rods, or "ghosts," are occasionally attached to extracellular spores.

After 24 hours the area of infiltration is slightly larger and is more sharply defined. The proportion of large mononuclear cells is increased, but polymorphonuclear leucocytes still predominate.

At the end of 2 days and subsequently an increasingly large part is played in the inflammatory reaction and in the phagocytosis of spores by the large mononuclear cells. The polymorphonuclear leucocytes in the meantime have either undergone disintegration or survive in small foci within the central parts of the infiltrated area. At the end of 2 days a large number of the spores have passed into the mononuclear cells, and by the end of 4 days the transference appears to be complete.

These large mononuclear cells correspond closely in distribution and behaviour to the polyblasts of Maximow (1902), and in modern nomenclature would be classed as histocytes (Aschoff, 1924). No evidence was found in support of their origin from the blood-cells. They appear in large numbers in close relation to capillaries in the periphery of the lesion. They are spherical, polygonal or lozenge-shaped. Their nuclei frequently show mitosis. In the resting stage the nuclei are spherical or reniform; they lie in an eccentric position and occupy from a half to a third of the total area of the cell. The nuclear membrane is sharply defined and is stained deeply in hæmatoxylin. The chromatin net is coarser than that of the fibroblast nucleus and is beset with three or more nodes of variable size. The cytoplasm is slightly spongy, slightly basophil and is stained less intensely than the nucleus in polychrome methylene blue. It was not always easy to differentiate these cells from the fibroblasts which also appear in considerable numbers at the periphery. The

possibility of transition taking place from one form to the other could not be excluded.

The centre of the lesion, from the 2nd day onwards, is mainly occupied by these large mononuclear cells. In this situation their cytoplasm becomes much more abundant and conspicuously foamy, and the cell outline is ill defined. Adjacent cells often appear to form a loose syncitium, threads of cytoplasm, which often contain small groups of spores, extending between the bodies of adjacent cells. An attempt to demonstrate fat or lipoid in the cytoplasm by staining frozen sections with Scharlach R was unsuccessful.

These cells are highly phagocytic and appear to have a greater affinity for the spores than do the polymorphonuclear leucocytes. Yet phagocytosis never appears to be complete: at all stages a small number of spores, perhaps 5 to 10%, lie outside the cell boundaries in section. The percentage of free spores is possibly much greater than this, but the indefiniteness of the cell outlines and the closeness with which the cells are often packed together make estimation difficult. Hence the percentages quoted are purposely conservative (see table).

In films prepared from the lesions the percentage of free spores always appeared to be greater than in the corresponding sections, and this discrepancy increased with the age of the lesion, that is with an increasing proportion of the large mononuclear cells to polymorphonuclear leucocytes. Two possible explanations of this discrepancy suggest themselves. First, free spores may be more readily picked up than cells in the free fluid which oozes from the cut surface, especially when the lesion becomes old and tough. Secondly, the amount of rubbing required to get enough material to make a film in these older lesions is probably sufficient to break off a number of cytoplasmic processes containing spores from the large mononuclear cells: the spores in these films frequently lie in groups closely connected with tags of cytoplasm. Support is lent to the second of these theories by the fact that when the infiltrating cells are mainly polymorphonuclear leucocytes the discrepancy observed is relatively slight.

(b) *Spores and earth inoculated.*—When a mixture of earth and spores is inoculated subcutaneously the initial infiltration with polymorphonuclear leucocytes is very much greater. The response is similar for both toxic and non-toxic spores. The mass of these leucocytes forms a central core which persists for a longer time than that resulting from the inoculation of spores alone. It is not appreciably altered in size by the end of the 6th day.

About this central core is a peripheral zone composed of the large mononuclear cells and spindle fibroblasts; by the 3rd day these cells are arranged round the core in a concentric manner. By the 6th day the peripheral zone is fully developed (Fig. 7). The large mononuclear cells are mixed in relatively small numbers with the polymorphonuclear leucocytes in the centre of the lesion; they collect in greater numbers in small knots about the junction of the central and peripheral zones. Here they are of larger size and have taken up large quantities of earth and spores.

Many spores and particles of earth are scattered throughout the centre both within and without the infiltrating cells. A greater proportion of the spores is extracellular than in corresponding sections where spores alone were

Experiment 2.—Table showing Vegetation and Approximate Degree of Phagocytosis.

Batch.		1st day.	2nd day.	3rd day.	4th day.	6th day.
I. Non-toxic spores	Film	40% spores extracellular; vegetation −	50% spores extracellular; vegetation −	60% spores extracellular; vegetation −	50–60% spores extracellular; vegetation −	80% spores extracellular; vegetation −
	Section	10% spores extracellular; vegetation −	5–10% spores extracellular; vegetation −	1–5% spores extracellular; vegetation −	5% spores extracellular; vegetation −	5% spores extracellular; vegetation −
II. Non-toxic spores + earth	Film	50% spores extracellular; vegetation −	10% spores extracellular; vegetation +	10% spores extracellular; vegetation +	20% spores extracellular; vegetation −	10% spores extracellular; vegetation ++
	Section	5–10% spores extracellular; vegetation −	5–10% spores extracellular; vegetation +	10% spores extracellular; vegetation −	10% spores extracellular; vegetation −	5% spores extracellular; vegetation +++
III. Toxic spores	Film	50–60% spores extracellular; vegetation −	20% spores extracellular; vegetation −	10–20% spores extracellular; vegetation −	60–70% spores extracellular; vegetation −	50% spores extracellular; vegetation −
	Section	5–10% spores extracellular; vegetation −	5% spores extracellular; vegetation −	5–10% spores extracellular; vegetation −	10% spores extracellular; vegetation −	5% spores extracellular; vegetation −
IV. Toxic spores + earth	Film	30–40% spores extracellular; vegetation −	10% spores extracellular; vegetation +	5% spores extracellular; vegetation ++	50% spores extracellular; vegetation +++	50% spores extracellular; vegetation +
	Section	50% spores extracellular; vegetation −	30–40% spores extracellular; vegetation −	30–40% spores extracellular; vegetation ++	20% spores extracellular; vegetation +++	10% spores extracellular; vegetation +++

inoculated. Thus in the central area 50% appear to be extracellular in guinea-pig IV (1), and from 30 to 40% in guinea-pig IV (3) (see table).

The correspondence between films and sections in respect of the number of free spores is more precise here than it was in the series where spores alone were inoculated. The cells in the films consist almost entirely of polymorphonuclear leucocytes.

In the central part of the abscess are seen small, ill-defined foci of irregular shape, occupied by fragments of disintegrated leucocytes mixed with particles of earth and free spores (Fig. 7). Vegetation of the spores is confined to these foci of necrosis, which are so small compared with the size of the abscess and so scanty that they may be absent from the half of the lesion taken for sections: for example, in guinea-pig II (3) bacilli were found in the film, but not in the sections of adjacent tissue (see table).

Slight vegetation was found on the 2nd day in guinea-pigs inoculated with a mixture of spores and earth, in both toxic and non-toxic groups. The vegetation reached its maximum on the 4th day in Batch IV (toxic spores) and on the 6th day in Batch II (non-toxic spores). In the latter batch, however, vegetation was missed both in films and sections on the 4th day, although it had been present on the previous day.

It has therefore been seen in Experiment 2 that when spores alone are inoculated subcutaneously into guinea-pigs vegetation does not occur, although phagocytosis is incomplete. When the spores are mixed with a heavy suspension of earth phagocytosis is still less complete. An abscess of considerable size is formed, and in restricted areas of this the spores form bacilli. Vegetation begins on the 2nd day and reaches its maximum 2 to 4 days later.

Experiment 3.—The later changes in lesions provoked by the subcutaneous inoculation of non-toxic tetanus spores and of a mixture of non-toxic spores and earth were traced further.

Two batches of 4 guinea-pigs were inoculated beneath the skin of the abdominal wall as follows:

Batch V: 0·2 c.c. suspension of non-toxic spores.

Batch VI: 0·2 c.c. suspension of non-toxic spores + 0·2 c.c. suspension of earth prepared as in Experiment 2 (p. 378).

One guinea-pig in each batch was killed after 8, 11, 14 and 17 days. The lesions produced were examined in the same manner as in the previous experiments.

Macroscopic Appearances of Lesions.

In Batch V the naked-eye appearances varied. On the 8th day the lesion was like a slight focal increase in the normal subcutaneous fat. Later a small, firm, sharply-defined white mass the size of a pin's head or millet-seed was found.

In Batch VI a definite greenish-brown, purulent area about the size of a small pea and with a soft core was found on the 8th day. On the 11th day and subsequently it had shrunk to the size of a lentil, becoming very firm and tough, with a homogeneous, yellowish-brown, cut surface.

Microscopic Appearances of Lesions.

(a) *Non-toxic spores alone.*—The histological appearances are essentially similar to those observed in the terminal stages of the corresponding series in Experiment 2 (p. 378). The lesion consists of a small compact mass of large mononuclear and spindle-cells. Large numbers of spores are packed within many of these cells. Extracellular spores are very rarely seen. Polymorphonuclear leucocytes are scarce and are usually restricted to a few small groups lying amongst the other cells in the centre of the lesion. They do not contain spores.

In the lesion produced after 11 days the tendency of the large mononuclear cells to coalesce is more marked than in previous stages (Exp. 2), partly perhaps because the mass is more compact, and the cells therefore lie more closely together. After 14 days the cells often appear to be fused to form rosettes or rings containing from 4 to 8 nuclei. Under a $\frac{1}{12}$-in. objective these groups of cells resemble foreign-body giant cells: numerous spores occupy those parts of the cells which form the centre of the rosettes or abut upon the lumen of the rings, while the nuclei are arranged at the periphery.

No vegetation is seen either in films or sections.

(b) *Non-toxic spores and earth.*—In the section taken 8 days after inoculation the lesion is smaller than that obtained after 6 days, but in other respects the characters are similar. Relatively few spores are found in the central area of polymorphonuclear leucocytes: between 10 and 20 % of these appear to be extracellular. After prolonged search through several sections one small necrotic focus was found in which a few bacilli, some of which appeared to be degenerating, were mixed with spores and *débris*. The greater proportion of spores and earth lie within the mononuclear phagocytes at the periphery of the central area.

In the later specimens vegetation is absent. The lesion becomes still smaller and poorer in polymorphonuclear leucocytes, while the large mononuclear cells encroach steadily on the central area. The spores and earth are engulfed by these cells; a very small number of spores appear to be extracellular. At 14 and 17 days giant-cell formation is very conspicuous.

Summary.—The subcutaneous inoculation of spores alone excites an inflammatory reaction, in which the polymorphonuclear leucocytes play the chief part in phagocytosis in the earliest stages. This phagocytosis by leucocytes is, however, transitory. From the outset large mononuclear cells, apparently derived from the tissues and in particular from the perivascular tissue, are present at the site of inoculation. They steadily increase in number and in size, and ultimately coalesce to form giant-cells. The spores are, with few exceptions, transferred to their cytoplasm and remain in this situation till at least the 17th day after inoculation. Phagocytosis is never quite complete: a few spores are always found outside cells in sections. The large number of spores which appear to be free in films of the older lesions probably results from the disruption of the mononuclear cells and giant-cells in the process of preparing the film.

There is no necrosis of cells in bulk in the inflammatory area, nor is there any evidence of vegetation of the spores.

When a *suspension of earth is added to the spores* there is a much greater inflammatory reaction, and a greater emigration of polymorphonuclear leucocytes. Necrosis of leucocytes occurs in small foci in the most central parts of the resulting abscess. Vegetation of the spores is confined to these foci. If toxic spores are introduced the animal dies as the vegetation reaches its maximum, that is on the 4th or 5th day after inoculation. If non-toxic spores are used the vegetation reaches its maximum at about the 6th day and then declines. After the 8th day no vegetating bacilli are present. The lesion at the same time shrinks, the leucocytes are gradually replaced by the large mononuclear cells, and an appearance resembling that produced by the inoculation of spores alone is acquired.

Phagocytosis again is always incomplete; the degree of incompleteness is greater in the earlier stages than in the later.

II. INTRAMUSCULAR INOCULATIONS WITH SPORES AND CALCIUM CHLORIDE.

With the object of obtaining lesions with a greater degree of vegetation, calcium chloride was adoped as the "tissue debilitant." This was suggested for this purpose by Bullock and Cramer (1919).

In order to avoid the risk of superficial sloughing a deep inoculation into the centre of the muscles of the thigh was made in preference to inoculation of the subcutaneous tissue.

Two batches of guinea-pigs were inoculated as follows:

Batch I: 6 guinea-pigs: 0·2 c.c. suspension of non-toxic spores + 0·5 c.c. of 5% calcium chloride.

Batch II: 3 guinea-pigs: 0·2 c.c. suspension of toxic spores + 0·5 c.c. of 5% calcium chloride.

One animal in each batch was killed subsequently at intervals of 1 day. In Batch I the guinea-pigs appeared healthy up to the time of death. In Batch II the second guinea-pig showed local tetanus and early general tetanus at the time at which it was killed, and the third guinea-pig died of tetanus towards the end of the 2nd day.

The skin was dissected off the muscles of the thigh. The whole block of muscle was then removed from the bone and fixed in 2% saline formaldehyde. After fixation the block was sliced transversely and suitable pieces taken for sectioning.

Macroscopic Appearances.

At the end of the 1st day the leg was intensely œdematous. The œdema subsided to a considerable extent in the later stages but was never absent. On transverse section there was a conspicuous yellowish-white opacity of a considerable portion of the central muscles of the thigh and the neighbouring areolar tissue. This change was maximal at the site of inoculation, but spread up and down the leg in the areolar septa involving a zone of adjacent muscle.

Microscopic Appearances. (Figs. 1 to 6 inclusive.)

The opaque areas observed with the naked eye in the muscle and areolar tissue show partial or complete necrosis and œdema. The area of necrosis is surrounded, in the early stages, by a dense fringe of polymorphonuclear

leucocytes from which discrete leucocytes pass inwards for a short distance leaving the greater part of the area free from infiltration. By the 2nd day a second and more peripheral zone of large mononuclear cells has appeared about the necrotic area. As a rule a band of non-infiltrated, or sparsely infiltrated, living tissue exists between these two zones (Fig. 4).

Vegetation, both of toxic and of non-toxic spores, has taken place with great rapidity and is of an exceptionally luxuriant character. It is confined to the necrotic areas, but extends into the borders of the zone of leucocytic infiltration. Phagocytosis of the bacilli can be observed along this line of contact. The bacilli lie in all parts of the necrosed muscle and are even found in compact groups in the centres of the fibres themselves.

At the end of the 1st day there is great vegetation and few spores are to be found. From the 2nd day onwards spores are not seen until the 5th and 6th days (Batch I). At these later stages the bacilli in the most central parts of the necrotic areas show loss of affinity for dyes and considerable spore-production. By the 6th day no bacilli are seen. All the necrosed muscle in the section (Fig. 6) has been invaded by polymorphonuclear leucocytes and these have engulfed all but a very small percentage of the spores.

Sections stained for calcium by Kossa's method show an abundant deposit of fine amorphous particles of calcium in the necrotic areas, and a still more abundant deposit at the periphery of the necrosed areas, in and between the two zones of infiltration. The particles are principally deposited within muscle-fibres; they are also present in the walls of arterioles and on the connective-tissue fibrils both of the muscle and the areolar tissue. In the later stages (Batch I: 5th and 6th days) there is conspicuous giant-cell formation by the mononuclear phagocytes about masses of these calcium particles.

There is no anatomical relationship between the distribution of the calcium and the bacilli in the sections. In general the distribution of the calcium is much wider than the area to which vegetation is restricted.

It appears, therefore, that vegetation of tetanus spores in the tissues of the guinea-pig takes place with much greater rapidity and intensity if a solution of calcium chloride is substituted for a suspension of earth in the inoculation. Microscopically this is correlated with the occurrence of large areas of necrosis which at first are devoid of leucocytes except at the extreme periphery. Vegetation is confined to these areas. The vegetation, which begins early and is of a very luxuriant character, reaches its maximum on the 2nd or 3rd day. At this stage guinea-pigs which have been inoculated with toxic spores die of general tetanus. If the guinea-pigs have been inoculated with non-toxic spores the bacilli degenerate and resporulate after the 4th day, and by the 6th day spores are the only form in which tetanus is present in the inoculated area.

There is no evidence that the calcium chloride of itself excites vegetation, for the distribution of calcium particles in the sections does not coincide with the distribution of the bacilli.

CONCLUSIONS.

1. The histological changes which follow the subcutaneous inoculation of toxic and non-toxic tetanus spores, with and without earth, into guinea-pigs

are described. Vegetation of the spores only occurred in those groups in which earth was employed. It was confined to small foci of necrosis in the centre of the infiltrated area.

2. When toxic spores were inoculated with earth the animal died at the height of vegetation. When non-toxic spores were used the vegetation reached a maximum within the same period of time, and then subsided within a few days.

3. Phagocytosis of the spores was always incomplete.

4. The substitution of calcium chloride for earth had a very stimulating effect upon the vegetation of the spores. This was correlated histologically with the presence of large areas of necrosis, to which the bacilli were confined.

DESCRIPTION OF PLATES.

All figures are semi-diagrammatic. Spores (red) and bacilli (blue) are introduced, regardless of proportions, to illustrate their distribution in the tissues. Degenerating bacilli are represented by blue outlines. Polymorphonuclear leucocytes are represented by fine black dots, large mononuclear cells by coarse black dots. Necrosed muscle is of a lighter tone than healthy muscle.

FIGS. 1–6.—Transverse sections of muscle and areolar tissue of thigh of guinea-pig following injection of 5% calcium chloride and spores (Exp. 3).

Fig. 1.—1 day after injection (toxic spores): a few spores and many bacilli in mass of necrosed muscle.

Fig. 2.—2 days after injection (toxic spores): many bacilli in sharply defined area of necrosed areolar tissue.

Fig. 3.—3 days after injection (toxic spores): bacilli in area of necrosed muscle.

Fig. 4.—4 days after injection (non-toxic spores): bacilli in area of necrosed areolar tissue; degeneration of bacilli in centre of this area.

Fig. 5.—5 days after injection (non-toxic spores): bacilli, some of which are degenerating, in necrosed muscle; resporulation beginning in intermuscular septum.

Fig. 6.—6 days after injection (non-toxic spores): resporulation complete in necrosed, infiltrated muscle.

FIG. 7.—Section of subcutaneous abscess obtained 6 days after inoculation of non-toxic spores and earth (Exp. 2): vegetation confined to a few small areas of necrosis in central core of polymorphonuclear leucocytes; spores scattered throughout central core and in adjacent zone of large mononuclear cells and fibroblasts.

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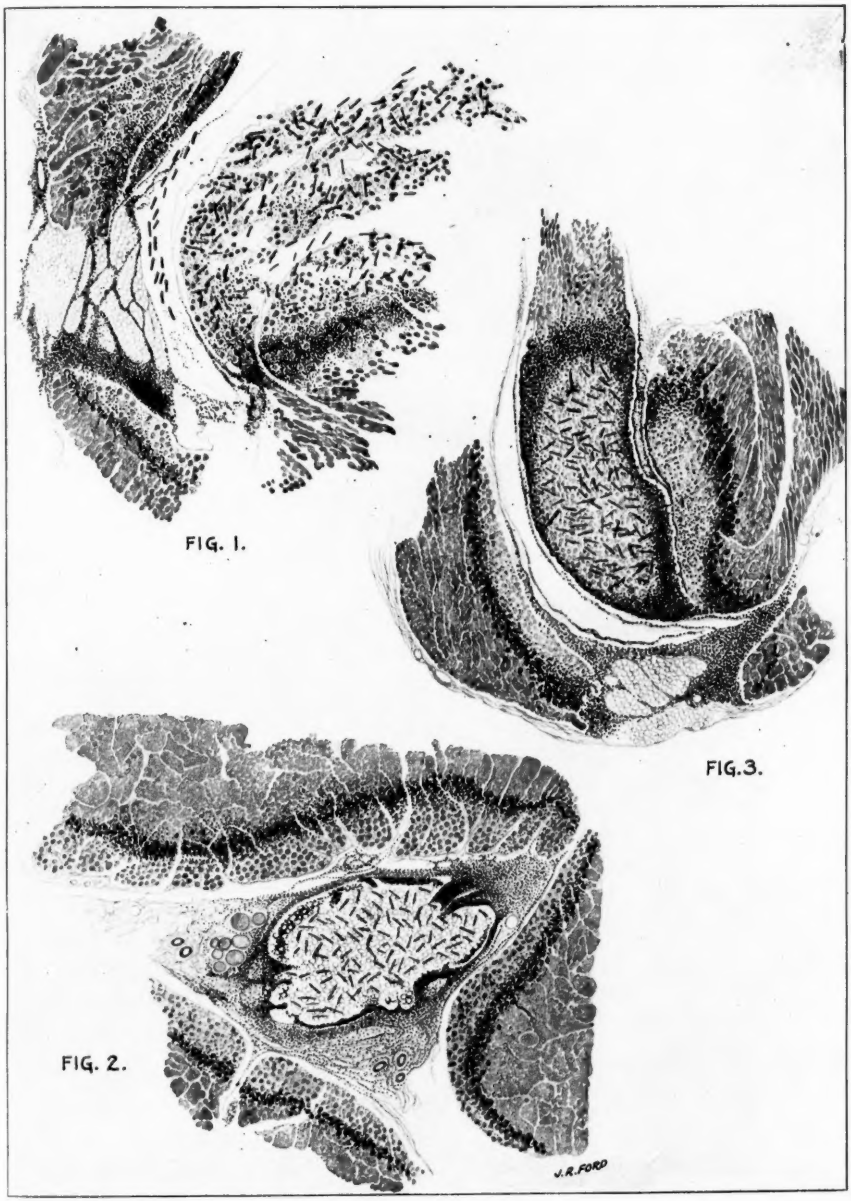
FIG. 1.
FIG. 3.
FIG. 2.
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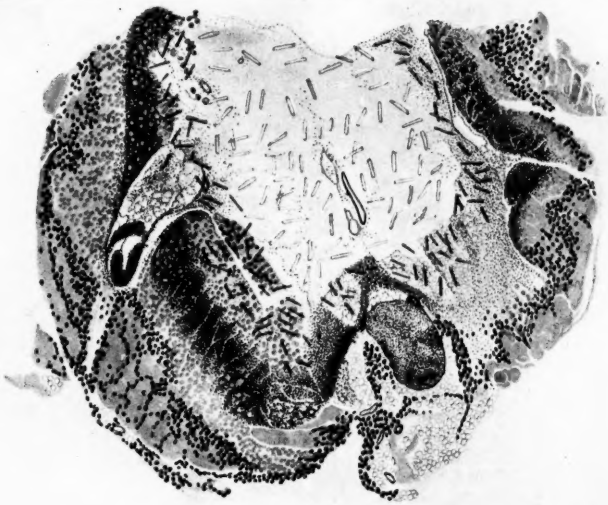
FIG. 4.



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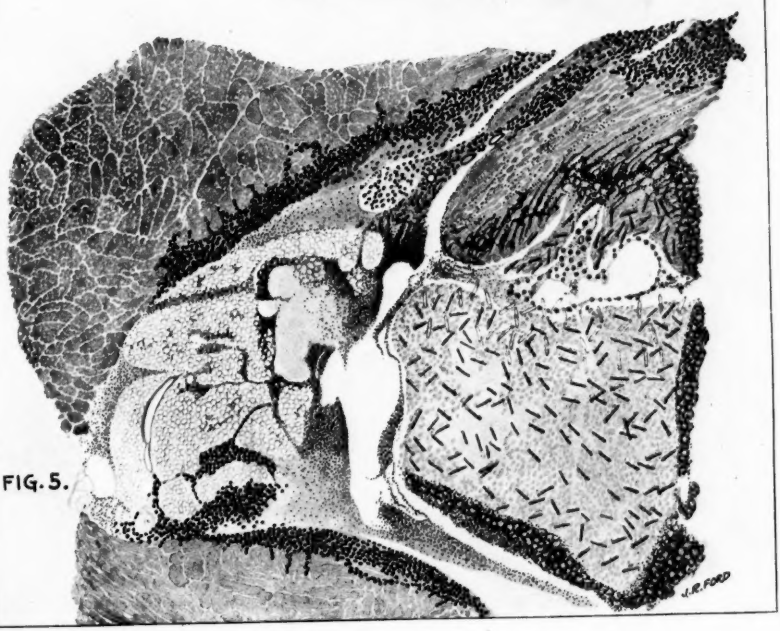

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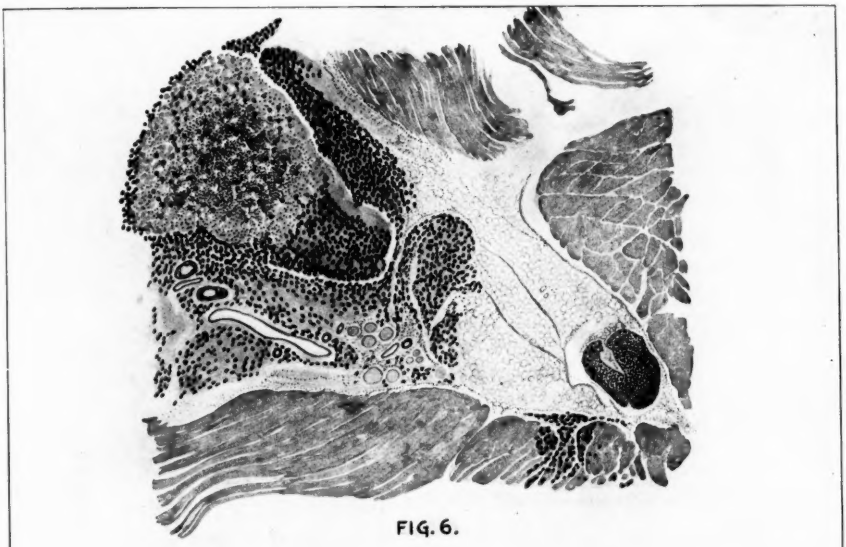

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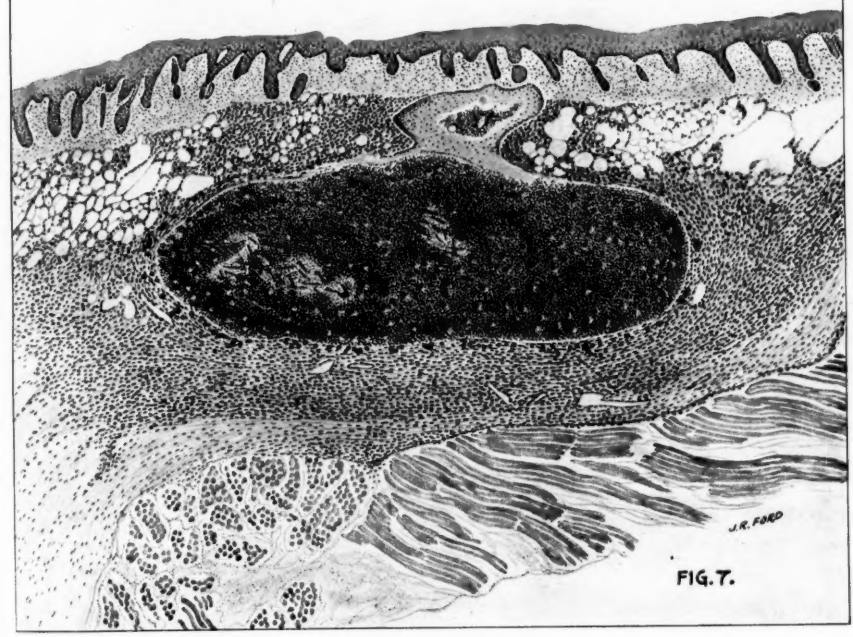

FIG. 7.

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TETANUS.—VI. THE CONDITIONS UNDER WHICH TETANUS SPORES GERMINATE *IN VIVO*.

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Received for publication July 31st, 1927.

In the classical papers of Vaillard and Vincent (1891) and Vaillard and Rouget (1892) it is shown that pure cultures of tetanus bacilli exert a pathogenic action only through the preformed toxin they contain. If these cultures are deprived of toxin by heat, the spores, though still viable, do not produce tetanus when inoculated aseptically.

These observations have been confirmed by all recent workers.

According to Vaillard and his collaborators the absence of pathogenic action is due to phagocytosis of the injected spores, and hence failure on their part to germinate and produce toxin.

This view has been generally accepted, although little confirmatory work has been carried out. A number of experiments have therefore been made to study the fate of spores when injected alone or together with tissue debilitants.

OBSERVATIONS ON THE RELATION OF PHAGOCYTOSIS TO VEGETATION.

Experiment 1: Subcutaneous injection.—Spores of tetanus bacilli were inoculated into a series of guinea-pigs and the local lesion examined by films at different intervals.

In all cases "spores" consisted of a 5-day surface growth of tetanus bacilli, washed up with a minimum of saline and heated to 65° C. for 1½ hours. The suspensions were then diluted with saline to a certain opacity approximating to 15 million spores per c.c. One c.c. of such a suspension inoculated subcutaneously or intramuscularly into guinea-pigs seldom produces any effect. On the other hand, on 4 occasions heavier suspensions have produced tetanus even when 2 years old. In view of the fact that the onset was more rapid than in "spore tetanus" and did not progress beyond the local stage, it is concluded that these cases were due to preformed toxin which had escaped destruction by heat, or to toxin which had been freed by maceration of the bacilli after heating. Vaillard and his collaborators drew attention to the occasional failure of the lower degrees of heat and recommended 80° C.

The inoculations (0·1 c.c.) were made with a fine needle through the depilated skin. Iodine was applied before the injection. In view of the known effect of bacteria on the vegetation of spores, it is necessary to make cultures of all lesions before conclusions are drawn, in order to exclude chance contamination. This is by no means rare when tissue debilitants are used.

The inoculum, although described as "spores," contains, of course, the attached bacillary bodies.

After intervals of 1, 2, 4, 20, 48 and 96 hours an animal was killed and examined.

The lesions were very inconspicuous, and hardly recognizable without the precise position of the needle-point being marked with Indian ink.

During the first 4 hours the spores were found in films of the inoculation area and a few leucocytes. No phagocytosis was observed. On the other

* Working under grants from the Medical Research Council.

hand, after 20 hours numerous phagocytes were present, and a number of these were charged with spores. Nevertheless, at this time many spores were found lying free in the films, still with the bacillary bodies attached. This persistence of the bodies is important. The body which remains attached to the spore after heating disappears with great rapidity when phagocytosed; in fact, it is exceptional to see a phagocytosed spore with body attached. The presence of the body, therefore, indicates that the spore seen has not been phagocytosed and then freed again by mechanical disruption in the making of the film.

In animals killed after 48 and 96 hours the picture is essentially similar, but now the attached bodies have become lysed. Though there is marked phagocytosis, there are also numerous free spores. No vegetation is seen at any time.

Experiment 2: Intraperitoneal injection.—0·5 c.c. of a suspension (5 million per c.c.) was inoculated intraperitoneally into a series of guinea-pigs, and the course of events studied by puncture of the peritoneum up to 24 hours after injection, and then by killing and examining the animal.

Leucocytes appeared in the exudate in about 15 minutes and the number increased up to 24 hours. Phagocytosis started in about 30 minutes and was practically complete in about 4 hours. In 24 hours the fluid contained many leucocytes but no spores. These were all congregated with masses of leucocytes, chiefly in the omentum. Many of the leucocytes in these masses were degenerate and undergoing phagocytosis by macrophages, together with the spores they contained. In 2 days in some cases one or two vegetating forms were seen, but on the third or subsequent days no vegetating forms have been met with. In about 30 % of cases guinea-pigs inoculated in this way have developed tetanus after a long interval, the shortest being 12 days and the longest 18.

The peritoneal route therefore leads to rapid and complete phagocytosis and sometimes tetanus, while the subcutaneous leads to delayed and incomplete phagocytosis without tetanus.

Many experiments have been carried out by injecting toxic spores and the non-toxic variants described by the author (Fildes, 1925, 1927), either alone, or with most of the tissue debilitants which have been described. The results of these experiments have been similar to those described by Russell (1927) in particular:

1. Germination of non-toxic spores takes place under the same conditions as of toxic.
2. Vegetation is transient.
3. The degree of vegetation depends upon the violence of the debilitant.
4. With a feeble debilitant only a very small proportion of the total mass of spores in a lesion germinate.
5. A variable proportion of the spores which do not germinate are not enclosed in phagocytes.

The demonstration that spores constantly remain for considerable periods in the tissues without undergoing phagocytosis, and without germinating, appears fatal to the suggestion that lack of germination is due to phagocytosis, and this is so however small the proportion of these spores may be. Vaillard

himself recognized the fact that a few spores were found free in films, but thought that this was due to rupture of loaded phagocytes during the process of film-making. This contention, in view of the presence of the attached bacillary bodies, is not sufficient to account for the majority of the free spores, at any rate in the first two or three days after injection. Russell (1927), however, is inclined to think that this factor has some influence in the later stages, although the occurrence of free spores in sections of the lesions shows that mechanical disruption is not the whole explanation.

It is, of course, not disputed that the lack of vegetation of phagocytosed spores may be due to the fact of phagocytosis, but obviously not the lack of vegetation of those spores which remain free.

THE RELATION OF GERMINATION TO NECROSIS.

The explanation of the fact that only a small proportion of the spores injected germinate is to be found in the work of Russell (1927). She has shown that germination takes place only in areas of necrosis which develop in the lesions produced by the debilitant.

The degree of necrosis depends upon the violence of the debilitant used. Sterile earth, for instance, merely produces occasional small areas among the masses of leucocytes, and the vegetating bacilli are strictly confined to these small areas. On the other hand, calcium chloride,* a more violent debilitant, produces a wide-spread necrosis, and throughout this area germination is complete.

According to the views of Vaillard and his collaborators, the action of sterile earth in determining germination would be ascribed to engorgement of the leucocytes with earth particles to such an extent that they cannot engulf the spores. Actually, however, the intensity of the phagocytosis of spores in these cases appears to be greater than when no earth is present. Vastly more leucocytes are involved. If it is admitted that germination takes place only in necrotic areas, then the school of Vaillard would argue that the invasion of leucocytes is prevented by these areas, and so germination takes place before the leucocytes have arrived, or, alternatively, that the influence which has produced the necrosis exerts a negative chemotactic action, or actually kills them and so disorganizes phagocytosis.

All these explanations may be correct, but nevertheless it is still necessary to explain why a spore lying free in a necrotic area at once breaks into active vegetation, while another spore lying free in a non-necrotic area remains in a totally passive condition.

THE OXYGEN TENSION OF THE TISSUES AND VEGETATION.

The contention of the present paper is that the germination of the spores of *B. tetani* is determined *in vivo* (as *in vitro*) solely by the tension of oxygen in the medium in which they lie.

Little attention has been directed to a possible effect of the oxygen tension of the tissues upon infections, but Novy and Soule (1925) have suggested that

* 0·5 c.c. of a 5% solution in water of $CaCl_2$ is the least quantity which will invariably activate spores in guinea-pigs.

the feeble growth of *B. tuberculosis* in caseating areas may be due to the low oxygen tension of such parts.

It will be recalled that in every patent capillary there is a definite tension of oxygen. The oxygen diffuses out of the capillaries, so that there is a falling gradient of tension in the tissues from that within the capillary to *nil*, if the intercapillary space is sufficiently wide. In this area of asphyxia substances are soon produced which occasion an active opening up of adjacent contracted capillaries, or a dilatation of the original capillary, both of which happenings produce again a positive oxygen tension in the area of asphyxia. Thus, in general, the capillary circulation may be looked upon as in a state of continual regional dilatation and constriction, which has the effect of producing in the tissues a tension of oxygen continually fluctuating from that in the capillaries to zero. At any one spot the zero tension is, of course, fleeting, otherwise the cells would be asphyxiated. Hence any area of intact tissue may be said to be constantly (though not permanently) under the influence of a positive oxygen tension.

Actual measurements made by Campbell (1924–25) show that in the subcutaneous tissues of the rabbit the tension of oxygen is 20–30 mm. Hg. Unfortunately the minimum tension of oxygen which will permit the germination of tetanus spores is not known, but it is common knowledge that germination will not take place in the presence of oxygen detectable by chemical means. Although when vegetation is actively taking place small quantities of oxygen can be coped with, it is generally thought that a complete absence of this gas is essential for germination.

This being so, the spore of a strict anaërobe implanted in intact tissues would not find itself in the correct gaseous environment.

In the case of an area of necrosis the respiratory conditions are clearly different. However small this area may be there will be a hindrance to the diffusion of oxygen, and a disturbance of the mechanism by which the capillaries are sympathetically dilated. The cells within this area will have consumed the local supply before themselves succumbing, and in the absence of renewal a permanent area of asphyxia will be established. In such an area any spore present will be under conditions suitable for germination and will germinate, but the bacilli will be unable to penetrate beyond the area of asphyxia to the area of positive oxygen tension.

PRODUCTION OF AREAS OF ASPHYXIA IN ANIMALS.

Attempts to produce areas of asphyxia in animals by interruption of the vascular supply are, of course, unavailing without interfering with the emigration of phagocytes. Nevertheless the indications of such an experiment are in favour of the present view.

Experiment 3.—Seven male guinea-pigs were anæsthetized with ether, the abdominal cavity opened and one testicle tied off with a ligature. Into each testicle 0·1 c.c. of a suspension of spores (15 million per c.c.) was then injected to a depth of $\frac{3}{16}$ in. The abdominal cavity was closed immediately, and after different intervals one of the animals was killed, namely after 2, 4, 6 and 24 hours. The three remaining guinea-pigs were found dead of

tetanus on the second day. This very rapid fatal result indicated an unusually active vegetation—even more active than when calcium chloride is used. Examination of sections cut from each testicle showed that this was the case. Whereas on the normal side no vegetation was seen at any time, on the ligatured side this was in active development even after 2 hours. There were more short vegetating forms than spores. In 4 hours the short forms had grown into filaments and had rapidly multiplied, but a few spores were still present. In 6 hours germination was complete and some of the organ infiltrated. In 24 hours the infiltration was still more marked, while in the animals which were found dead on the second day, the whole testicle was permeated with bacilli which were already in an active state of sporulation.

Ligation of the vessels has, therefore, the effect of inducing a profound germination, but at the same time it interferes with the emigration of leucocytes. In the 2-hour specimen, however, in which there was marked vegetation on the ligatured side, phagocytosis by leucocytes had hardly commenced on the normal side. The capillaries were engorged with leucocytes and emigration had started, but the cells were still congregated about the capillaries. A considerable degree of phagocytosis was, however, already present chiefly by tissue-cells, but some 20–30 % of the spores were still obviously free. After 6 hours a smaller number of spores remained free on the normal side, but after 24 hours phagocytosis appeared to be complete.

It is clear from this experiment that a profound vegetation is in existence on the ligatured side at a time when phagocytosis is undoubtedly incomplete on the normal side, and it seems probable that the asphyxia produced is the deciding factor in the vegetation. It has, however, been suggested that in severe injuries and necroses the tissues become in some way broken down or altered into a medium more satisfactory for growth. In the testicle, however, in the first 6 hours there is no obvious histological evidence of necrosis at all. Thus, while it may be admitted that the initial stages of necrosis are already in progress after 2 hours, gross histological necrosis is not a necessary requirement in germination.

DISCUSSION.

If the tension of oxygen in the tissues is the cause of the lack of germination of inoculated tetanus spores, it follows that Vaillard's concept of the protective action of phagocytosis must be abandoned in this respect. Whereas in his view "the body is not an indifferent medium, but defends itself," the present view suggests that the body is a passive agent in this matter. It may even be supposed that the activity of the phagocytes is apt to further germination rather than hinder it. In the experiments already referred to in which tetanus followed intraperitoneal inoculation of spores, this result is clearly due to the great concentration of leucocytes and spores in the omentum, where veritable abscesses are formed, with resultant necrosis and its effects on oxygenation. The condition is the same, though less marked, as after injection of aleuronate.

Even in the natural infection phagocytosis of spores does not appear to

play any effective *rôle*. The process may be more complete than in the experimental owing to the much smaller dose of spores, but there is little reason to suppose that phagocytosis has any destructive action on spores, at least for a long time. The differences in staining reaction remarked on by Vaillard are paralleled by the spores in a simple suspension, and it is well known that they may be recovered from the local lesion months after inoculation.

The fact of phagocytosis seems to be a matter of indifference. The spores are as likely to become involved in an area of asphyxia if they are phagocytosed as if they are not, since in each case they are intimately mixed with the bacteria and foreign matter to which these areas may be ascribed.

If any protective action is exerted by phagocytosis in tetanus, it seems more probable that this would operate by destroying the accompanying bacteria and so preventing necrosis.

Bullock and Cramer (1919), working on gas-gangrene, considered the possibility of the debilitant forming "conditions favourable to anaërobiosis," and rejected the possibility on the following grounds:

1. The same conditions favour aërobic bacteria.
2. *B. welchii* and *V. septique* invade the blood-stream.
3. Section of the femoral artery must "produce a profound deficiency in the oxygenation of the tissues of the leg," but does not "make the limb a more suitable soil for the experimental production of gas-gangrene."
4. Lactic acid produces necrosis but no activation of gangrene spores in mice.

If Bullock and Cramer's conclusions on gas-gangrene are used to oppose the present conclusions on tetanus, it may be remarked that:

1. The organisms used were streptococci, which are often preferably anaërobic.
2. *B. tetani* neither invades the blood-stream nor spreads from the local lesion *in a vegetating form*, although, of course, spores are distributed to a slight degree.
3. Section of the artery would not produce such a deficiency as to approximate to asphyxia of the limb.
4. I have not worked with lactic acid in guinea-pigs. This substance has, of course, often been used to activate tetanus spores in guinea-pigs.

The two diseases have, however, so many points of difference that it is doubtful whether Bullock and Cramer's results can justly be applied to tetanus.

CONCLUSIONS.

1. Phagocytosis is not the cause of the absence of germination of inoculated tetanus spores.
2. This is due to a positive tension of oxygen in the tissues.
3. In necrotic areas the tension of oxygen may fall to zero and any spores therein will then germinate.

I am indebted to Dr. H. W. Florey for carrying out the operations on guinea-pigs.

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TETANUS.—VII. THE TREATMENT OF TETANUS IN RABBITS BY LARGE INTRATHECAL DOSES OF ANTITOXIN.

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Received for publication September 9th, 1927.

THERE is still a divergence of opinion as to the value of intrathecal administration of antitoxin in the treatment of tetanus.

Sherrington (1917) injected 8 M.L.D. toxin into the calf of monkeys, and two or three days later when local tetanus was well marked and trismus sometimes present, treated them by various routes with one dose of about 3 c.c. of antitoxin.

Among other results he found that 7 out of 25 monkeys recovered after intravenous injection and 14 out of 25 after lumbar intrathecal. The injection was made after operative display of the meninges and so undoubtedly entered the theca.

Park and Nicoll (1914), working with guinea-pigs, used 2 M.L.D. toxin, and treated the animals about 19 hours afterwards, when local tetanus was present, with different quantities of antitoxin. They considered that their results "would seem absolutely conclusive of the superiority of the intraspinal method of giving antitoxin over the intravenous route."

Analysis of these shows that with intravenous injection of 250 units, *i.e.* about 750 units per kilo body-weight, 3 out of 5 animals died; after 200 units 2 out of 3 died, and after 100 units 5 out of 5. On the other hand, after intrathecal injection of 250 units only 2 out of 8 died. They did not treat any animals with 100 units, but with 50 units 1 out of 4 died. The most surprising result, however, is that with 10 units no deaths occurred in 4 cases. Thus, while by the intravenous route a diminution in the amount of serum led to fewer recoveries, intrathecally it resulted in more recoveries.

Incidentally Park and Nicoll made their injections by cutting down on to the spine and then inserting a fine needle into the theca.

* Working under grants from the Medical Research Council.

Preliminary experiments to those about to be described, however, showed that the space in a large guinea-pig into which a needle has to be put to effect an intrathecal injection is very small indeed—from 1 to 2 mm. in length. The thecal space occupies only a portion of the bony canal, the space between the dura and bone carrying very large venous sinuses.

In some of our experiments in which 25% salt was run into the saphenous vein and horse-serum coloured with trypan-blue allowed to pass into the "theca" from a burette, astonishing amounts were injected. In one guinea-pig of 750 gm., 11·55 c.c. of serum were run in in 22 minutes after 3·1 c.c. of 25% salt solution intravenously. In another animal 7·76 c.c. in 40 minutes. In the animals blue serum was found filling the subarachnoid space, even covering the cerebral hemispheres, but the following experiment gave a clue as to where the bulk of the injected serum was going.

The spinal needle was inserted in the usual manner and the serum allowed to flow before any injection of salt. In three minutes 5·2 c.c. serum had been run in, which killed the animal. On examination no trace whatever of blue serum could be found except a faint staining round the needle-point. The only reasonable explanation is that the serum had been injected into one of the venous sinuses.

Even when the dura was exposed by a laminectomy, the performance of which occasioned considerable hæmorrhage from the venous sinuses before mentioned, it was found impossible to be sure that a measured quantity of serum was introduced into the theca, owing to leaks back along the needle.

We do not wish it to be inferred that intrathecal injections cannot be made in the guinea-pig, but insist that one cannot be sure that in every instance the injection fluid passes into the subarachnoid space, and not into the space between the dura and bone or into the venous sinuses.

We have come across only two other satisfactory attempts to determine the value of the intrathecal route. Permin (1914) could find no difference between the intravenous and intrathecal routes in goats. Gottlieb and Freund (1916) found that after 3 M.L.D. in rabbits 1 c.c. antitoxin intravenously failed to prevent death even 6 hours after the toxin injection, while intrathecally (bulbar) it was effective up to 25 hours.

EXPERIMENTAL.

The experiments to be recorded here were carried out on rabbits, a procedure being adopted whereby the cerebro-spinal fluid was almost, if not completely, replaced by antitoxin.

The following considerations were in mind at the outset of the experiments*:

The cerebral capillaries are apparently very impermeable to protein, and react in different ways from those present in other organs. Some analyses of rabbit cerebro-spinal fluid (Hewitt and Florey) have shown that the normal protein content is about 0·03%. It would seem reasonable, therefore, to suppose that antitoxin, which, if not a protein, is attached to one, would find

* Ebaugh (1922) in the treatment of cerebral syphilis, and Aycock and Amoss (1924) in that of infantile paralysis, have utilized the same principle.

considerable difficulty in passing from the blood-stream into the spaces surrounding the nerve-cell, *i.e.* in applying itself to the seat of action of the tetanus toxin.

The intrathecal route for the administration of antitoxin was first devised in the belief that the antitoxin would be more directly applied to the nervous system. Weed (1922) and his associates have shown that the bulk of the cerebro-spinal fluid comes from the choroid plexus, a small amount also passing out along the perivascular spaces to the subarachnoid space. From the subarachnoid space the fluid passes through the arachnoid villi into the venous sinuses. That is to say, fluid introduced into the theca normally passes out through the arachnoid villi and not into the nervous tissue. Weed also showed that the intravenous administration of hypertonic salt solution was capable of reversing the flow in the perivascular spaces—the fluid from the arachnoid space being sucked in around the pericapillary spaces (this we have verified), and even into the perineuronal spaces.

With these facts in view it seemed possible to administer the antitoxin so that it penetrated *into* the nervous tissue.

The rabbit was selected for the experiments after abortive attempts in guinea-pigs and cats. As already indicated, we were unable at any time to be certain that the serum entered the subarachnoid space in guinea-pigs, and therefore we abandoned this animal in favour of the cat. The operation in the cat was, of course, satisfactory, but this animal is so little susceptible to tetanus toxin that consistent results could not be obtained.

We then had recourse to the rabbit. The objection to this animal is that it is a poor operative subject, and a number of animals failed to survive the somewhat extended manipulations. It was difficult to achieve a complete series from which to draw conclusions.

The following technique was eventually evolved for administration to rabbits:

Intravenous urethane, 0·75 gm. per kilo. supplemented by ether, was used as an anæsthetic. The rabbit, back upwards, was tied out on a board supported on a pillar. The head, gripped in a clamp, was flexed so as to put the back of the neck on the stretch. This area was shaved and prepared for aseptic operation. A longitudinal incision was made down to the atlanto-occipital membrane, which was well exposed. The antitoxin was contained in a burette furnished with a Marriotte tube. This burette was connected to a hypodermic needle, the point of which was ground less obliquely than is usually the case. In order to avoid running cold fluid on to the medulla, this hypodermic needle was fitted with a small collar containing an electrical resistance. By connecting to a 4-volt battery and a slide-wire resistance the temperature of the drops issuing from the point of the needle could be regulated. The hypodermic needle was pushed through the atlanto-occipital membrane as far as to ensure the point being free in the subarachnoid space.

When this portion of the apparatus had been satisfactorily adjusted a second burette containing 25% sodium chloride solution was attached to a hypodermic needle, which was inserted into an ear vein. The rate of flow from this was usually adjusted to be about 1 c.c. per minute, and usually 15 c.c. were injected. The effect of this hypertonic salt solution on the serum

soon became manifest, as after a first sharp inrush following the release of the cock (due to bulging of the atlanto-occipital membrane) there was a steady passage of serum into the subarachnoid space. 2 c.c. serum (1000 units) was usually run in in about 30 minutes. After this amount of antitoxin had been run in the flow was stopped, the needle removed from the subarachnoid space and the wound closed. After 2 hours normal saline was administered intraperitoneally, as the animal passed large quantities of urine and became very dehydrated after the hypertonic saline.

As in most rabbits it is impossible to extract more than 1 c.c. of cerebro-spinal fluid, it seems reasonable to suppose that the injection of 2 c.c. antitoxin had almost replaced the normal cerebro-spinal fluid.

Rabbits, without tetanus, treated in this way recovered completely, so that we can confidently assert that the treatment was not of itself a cause of death.

The rabbits used for ascertaining the efficacy of the treatment were given 4 minimum lethal doses of a liquid tetanus toxin into the triceps muscle of the fore limb. The first signs of local tetanus usually appeared in 24 hours, namely a tendency more or less marked to flexion of the paw. The spasticity rapidly increased, so that in 2 days the whole limb was stiff, drawn back and often adducted across the thorax. At this time, also, the head was beginning to be turned and faced to the inoculated side. In an untreated animal the usual symptoms rapidly progressed and death took place on the third day. The actual times of onset of the various stages were, of course, subject to considerable variation in different animals—a fact which must be taken into consideration when carrying out the experimental treatment.

Treatment was given on the second day after injection of the toxin, the test animal and the control treated with the same quantity of antitoxin intravenously being selected as being in the same stage of tetanus.

The treatment was carried out successfully on 10 rabbits, with the result that when the signs at the time of treatment had not progressed beyond a stiffness of the leg, all recovered (whether with intrathecal or intravenous administration), but when there was the slightest sign of involvement of the head all died, though not quite so quickly as the untreated controls.

Thus there was no evidence that our method had any advantage over a simple intravenous injection.

In view of the relative success of the intravenous treatment, it was thought possible that the intravenous injection of urethane might have altered the permeability of the cerebral capillaries and allowed passage of antitoxin. In sufficient concentration urethane is capable of causing stasis in capillaries. This, however, was not the case, as rabbits in which the neck muscles were slightly affected died whether they received intravenous antitoxin after a preliminary injection of urethane or not.

CONCLUSION.

The results of our experiments do not show that the administration of large doses of antitoxin intrathecally, with considerable penetration of the nervous tissues, has, in rabbits, any greater value than the administration of the same quantity of antitoxin intravenously.

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THE ADHESION PHENOMENON AS AN AID TO THE DIFFERENTIATION OF LEPTOSPIRA.

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Received for publication September 13th, 1927.

IN a previous paper (Davis and Brown, 1927), the phenomenon of adhesion, also known as the thrombocytobarin or Rieckenberg reaction, was described in its application to the differentiation of trypanosomes. We now propose to submit the results of experiments regarding the applicability of this reaction to the differentiation of the various types of leptospira.

It may be recalled that the test consists in allowing an immune serum to interact with the specific organism at an appropriate temperature in the presence of a suitable indicator of a visible particulate nature. At the expiration of a short interval a drop of the mixture is examined by means of dark-ground illumination, and a positive result is characterized by the adherence of the indicator particles to the organism.

We began this work by inoculating a number of rabbits with cultures of the various strains of leptospira then available. The doses given intraperitoneally at weekly intervals consisted of approximately 5 c.c. of a well-grown culture in Wenyon-Noguchi medium.

The following strains were used:

1. *Leptospira biflexa*, Wolbach and Binger, *i.e.* the free-living type, the *pseudo-icterogenes* of Uhlenhuth; this had been isolated in pure culture from London tap-water in 1925.

2. *Leptospira icterohæmorrhagiæ*, Inada and Ido, isolated in 1922 from Dr. Manson Bahr's case, the patient being a seaman who developed spirochætal jaundice one week after having fallen into the Thames.

3. *L. icterohæmorrhagiæ* (Rat 1), isolated in February, 1926, from a London wild rat.

4. *L. icterohæmorrhagiæ* (Rat 2), isolated in October, 1926, from a similar source.

Rat 2 strain was pathogenic to guinea-pigs, and was therefore killed by heating to 56°C. for ½ hour before inoculating. The other strains of *L. icterohæmorrhagiæ* had lost their virulence.

The sera were tested against the original strains, together with other strains derived from natural waters, jaundiced dogs, and a tame rat. The water strains 2 and 3 had been maintained in fæcal cultures (Hindle, 1925).

The technique of the test is essentially as follows: a small measured volume of the fresh antiserum is mixed in an agglutination tube with an equal volume of culture fluid containing the leptospira. The most suitable type of culture is one having numerous discrete leptospira in a free-swimming state. To the mixture is added a volume of a thin bacterial suspension in saline. After incubating the mixture at 30°C. for 20 minutes a drop is examined by dark-field illumination. In a strongly positive reaction all the

Positive reaction. Negative reaction.

Drawing from a dark-field preparation of the adhesion phenomenon as applied to *L. bifleza*.

leptospira are seen to be individually enveloped by adherent bacteria. Weaker positive results may show a varying percentage of the leptospira unaffected; a negative reaction is indicated by the absence of any adhesion of bacteria to the leptospira.

Table I shows that the strains from human, rat and dog infections behave similarly, but that the free-living water strains act quite distinctly. This result accords with the agglutination experiments of Uhlenhuth and Zuelzer (1920).

These experiments have been repeated many times with consistent results.

Antisera have been successfully prepared in rabbits, guinea-pigs, rats and mice. During the last few months, however, we occasionally found that antisera prepared from the Weil or animal strains acted non-specifically, in that they gave a weak reaction with the water leptospira. This occasioned us to examine the sera of a number of stock laboratory animals, with the result that several were found to give a positive adhesion reaction to the water strain. We suggest that this is due to antibody formation as a result of the ingestion of these organisms. Feeding experiments are in progress with a

view to testing this contention. None of the animals were found to react serologically with any other type of leptospira.

These findings necessitate the preliminary serological examination of animals when it is desired to prepare specific sera to leptospira other than of the water type; further, during the period of immunization precautions should be taken to eliminate the presence of *L. biflexa* in the animal's food-supply.

TABLE I.

Strain.	Anti-serum. *L. biflexa* (water).	Anti-serum. *L. icterohæmorrhagiæ*. Human.	Anti-serum. *L. icterohæmorrhagiæ*. Rat 1.	Anti-serum. *L. icterohæmorrhagiæ*. Rat 2.	Control: Normal rabbit serum.
L. biflexa (water) 1	+	0	0	0	0
2	+	0	0	0	0
3	+	0	0	0	0
L. icterohæmorrhagiæ:					
Human	0	+	+	+	0
Rat 1	0	+	+	+	0
Rat 2	0	+	+	+	0
Dog 9	0	+	+	+	0

When, as is subsequently to be described, normal guinea-pig's serum is used to reactivate old specific antisera, care should likewise be exercised to exclude any such natural antibody factor.

During the past eight months the sera of one hundred London wild rats have been examined for adhesion antibodies. These rats have been examined weekly in batches of four; their sera were tested against cultures of *L. icterohæmorrhagiæ*, human and rat types, and of *L. biflexa* maintained in Wenyon-Noguchi medium.

Of the hundred rats, 32 gave positive reactions to both the human and rat types, none reacted with human or rat types alone, 4 reacted positively with human, rat, and water strains, and 7 gave positive reactions with *L. biflexa* alone.

It is of interest to compare these results with those of Stevenson (1922) and Buchanan (1927), who examined wild rats in London and Edinburgh respectively for the presence of leptospira. The former reported 30%, and the latter 36% infected.

Langworthy and Moore (1927) have recently examined wild rats in Philadelphia for agglutinating antibodies to leptospira, and report their presence in 60% of the animals examined.

In May, 1927, we were able through the kindness of Dr. Noguchi and Prof. Ledingham to secure cultures of five strains of *L. icteroides*, Noguchi, and one strain of *L. hebdomadis*, Ido, Ito and Wani, the supposed causal organisms of yellow fever and the seven-day fever of Japan respectively. Their reactions with the antisera in our possession were investigated, and with the serum of a wild rat, No. 42, which had given a strongly positive reaction with *L. icterohæmorrhagiæ*. As will be seen from Table II, the various strains of *L. icteroides* reacted with the human and rat antisera, but not with the water antiserum. *L. hebdomadis* did not react with any of these sera.

TABLE II.

Strain.	Anti-serum. *L. biflexa* (water).	*L. icterohæmorrhagiæ.* Human.	*L. icterohæmorrhagiæ.* Rat 1.	Serum of wild rat No. 42.
L. biflexa (water) 1	+	0	0	0
2	+	0	0	0
3	+	0	0	0
L. icterohæmorrhagiæ:				
Human	0	+	+	+
Rat 1	0	+	+	+
L. icteroides:				
Guayaquil strain, Cedeno 6	0	+	+	+
Guayaquil strain, Chatin 5	0	+	+	+
Brazilian strain 3	0	+	+	+
Peruvian strain 2	0	+	+	+
Le Blanc strain	0	+	+	+
L. hebdomadis	0	0	0	0

A series of white rats and of mice were each given three injections, at weekly intervals, of all the strains of leptospira now available. At the expiration of the fourth week the animals were bled, and their sera tested against cultures of the various strains. The results are given in Table III. It may be recorded that the strains *L. icteroides* and *L. hebdomadis* were not pathogenic to guinea-pigs.

TABLE III.

Strain.	Anti-serum. *L. biflexa* (water).	*L. icterohæmorrhagiæ.* Human.	*L. icterohæmorrhagiæ.* Rat.	*L. icteroides.* Guayaquil, Cedeno 6.	*L. icteroides.* Guayaquil, Chatin 5.	*L. icteroides.* Brazilian 3.	*L. icteroides.* Peruvian 2.	*L. icteroides.* Le Blanc.	*L. hebdomadis.*
L. biflexa (water)	+	0	0	0	0	0	0	0	0
L. icterohæmorrhagiæ:									
Human	0	+	+	+	+	+	+	+	0
Rat	0	+	+	+	+	+	+	+	0
L. icteroides:									
Guayaquil strain, Cedeno 6.	0	+	+	+	+	+	+	+	0
Guayaquil strain, Chatin 5.	0	+	+	+	+	+	+	+	0
Brazilian strain 3	0	+	+	+	+	+	+	+	0
Peruvian strain 2	0	+	+	+	+	+	+	+	0
Le Blanc strain	0	+	+	+	+	+	+	+	0
L. hebdomadis	0	0	0	0	0	0	0	0	+

It will be seen that the leptospira are clearly differentiated into three groups: viz. *L. biflexa*, which has behaved throughout as a distinct serological type; secondly, the human and rat strains of *L. icterohæmorrhagiæ* and the various strains of *L. icteroides*, all of which react as a homogeneous type; and thirdly, *L. hebdomadis*, which is quite distinct from the above two groups.

In our previous paper reference was made to the work of Kritschewski and Tscherikower (1925), who, working with trypanosomes, found that the antibody was thermostable, but that the reaction depended upon the presence of

free complement. Owing to the difficulty of removing the last traces of complement from the trypanosome suspension without injuring the organism, we could not satisfy ourselves regarding this point. With leptospira, however, we had no difficulty in demonstrating that the presence of complement is essential for the reaction to take place.

It was found that minute quantities were sufficient. A guinea-pig's serum in which the minimum hæmolytic dose of complement was 1 in 45, when diluted to a total concentration of 1 in 900 was sufficient to reactivate an anti-leptospiral serum which had been heated to 56°C. for 30 minutes. Antiserum which had been kept for two years at room temperature was capable of being reinactivated by the addition of fresh complement; in this case the serum had been prepared at the Wellcome Physiological Laboratories for veterinary use.

Although the titre of the antibody prepared in rabbits varies considerably, it is not usual to obtain a serum which will react positively after being diluted more than ten times with saline; this, mixed with one volume of the culture of leptospira, together with an equal volume of the bacterial suspension, would give a total concentration of 1 in 30.

In the course of observations on heated sera a specific lytic effect was found to be present which was not in evidence when using the fresh serum; this finding is of interest in view of the work of Schüffner and Mochtar (1927[1]), who pointed out that this specific lysis of leptospira takes place in the absence of complement, and very often only becomes evident when the serum is considerably diluted.

DISCUSSION.

The results obtained in the present investigation indicate that the adhesion phenomenon is a simple serological reaction which renders possible the differentiation of certain types of leptospira. Owing to the extensive divergence of opinion which exists regarding the identity or otherwise of *L. icterohæmorrhagiæ* and *L. icteroides*, the results obtained by the employment of this reaction are of interest. Noguchi (1925) maintains that these two organisms are not only distinct from a serological and immunological point of view, but also affirms that they can be differentiated on morphological grounds.

During the epidemic of yellow fever which he investigated in Guayaquil he found that 67% of the local wild rats were infected with *L. icterohæmorrhagiæ*, but that antisera prepared from the strains isolated from these rats would not noticeably protect guinea-pigs against the strains (*L. icteroides*) isolated from human cases of yellow fever (Noguchi, 1919).

Wenyon and Brown, in the course of the investigation of the leptospira which they isolated from Manson-Bahr's case of the seaman who fell into the Thames, found that the serum of this patient would protect guinea-pigs, not only against the reaction produced by the inoculation of cultures of *L. icterohæmorrhagiæ*, but also against similar febrile reactions caused by the injection of two strains of *L. icteroides* (Manson-Bahr, Wenyon and Brown, 1922).

More recently Battistini (1925), Theiler and Sellards (1926), Schüffner and Mochtar (1927[2]), Puntoni (1927), and Sellards (1927), using various serological and immunological tests other than the adhesion phenomenon, have

unanimously come to the conclusion that the two organisms are identical serologically.

It is beyond the scope of this paper to discuss the ætiological relationship of *L. icteroides* to yellow fever; all that can be affirmed is that the adhesion phenomenon affords additional evidence of the serological identity of *L. icteroides* and *L. icterohæmorrhagiæ*.

The actual virulence of the organism at the time of examination does not appear to affect the result, for with the exception of one rat strain of *L. icterohæmorrhagiæ* all had lost their pathogenicity for guinea-pigs.

In view of the commonly held theory that the transformation of the free-living *L. biflexa* into the pathogenic *L. icterohæmorrhagiæ* may take place in the tissues of the wild rat, the prevalence, in our series of wild rats, of antibodies to *L. icterohæmorrhagiæ* is not without interest. That such a transformation may occur is supported by the recent work of Uhlenhuth and Hermann (1927), who, after blockading the reticulo-endothelial systems of young wild rats, injected *L. biflexa*, and subsequently recovered a pathogenic strain. We have shown that the presence of antibodies to *L. biflexa* is relatively common in guinea-pigs, rabbits and white rats, yet such antibodies are infrequently found in wild rats—11% in our series; whereas antibodies to *L. icterohæmorrhagiæ*, which we never found in laboratory animals, were present in 36% of the wild rats. The possibility presents itself that in the small percentage of wild rats, the sera of which react with both types of leptospira, the transformation may be in progress.

In our opinion this reaction possesses distinct advantages over the agglutination reaction as applied to leptospira, in that the result, which can be obtained in a much shorter time, can be more easily read with certainty.

SUMMARY.

1. The application of the adhesion phenomenon to the differentiation of leptospira has been described. By its aid the various strains fall into three distinct groups: the free-living or water type to which we have referred as *L. biflexa*; the Weil and yellow fever type, *L. icterohæmorrhagiæ* and *L. icteroides*, which behave identically; and lastly, the causal organism of the seven-day fever of Japan, *L. hebdomadis*.

2. The sera of one hundred London wild rats have been examined for adhesion antibodies to human, rat, and water strains of leptospira.

3. A considerable number of stock laboratory animals have been found to possess adhesion antibodies to the water type of leptospira.

4. The statement of Kritschewski and Tscherikower that the adhesion antibody is relatively thermostable but dependent for its action upon the presence of thermolabile complement has been confirmed. Further, it has been shown that the presence of minute quantities of complement are sufficient for the reaction.

We wish to acknowledge our indebtedness to Dr. C. C. Okell and Dr. St.John Brooks for kindly supplying us with several of the cultures used in this investigation, and to Mr. B. Jobling for the accompanying illustration.

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THE APPLICATION OF THE RAMON FLOCCULATION TEST TO THE TOXIN AND ANTITOXIN OF *STREPTOCOCCUS SCARLATINÆ.*

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Received for publication August 3rd, 1927.

THE difficulties in the way of standardizing scarlet fever toxin and antitoxin due to lack of an adequate supply of suitable human volunteers is too well known by workers in this field to require further comment, and has led to the introduction of several means of superseding the skin-test method. Kirkbride and Wheeler (1926) have published results using a skin-test in goats, but their work has, as yet, received no confirmation. Dochez and Sherman (1925) have made use of a skin-test in sensitized guinea-pigs. Birkhaug (1925) with erysipelas toxin and antitoxin has elaborated a skin-test in rabbits using whole cultures, and Okell and Parrish (1927) have shown that by the use of intravenous inoculation of cultures of Dochez's strain, following an earlier injection of scarlet fever antitoxin, a fair idea of the protective value of a serum can be obtained. Since in scarlet fever we are

dealing with a definite toxin for which a corresponding antitoxin can be prepared, it seemed advisable to discover if the flocculation test of Ramon (1922) would prove of value in determining the unitage of scarlet fever toxin and antitoxin. Some attempt has already been made in this way by Dyer (1925), with promising results. It was accordingly decided to test for ourselves the value of the test in its application to scarlet fever.

EXPERIMENTAL WORK.

An antitoxic horse-serum that had been prepared for clinical use was obtained and its neutralizing value ascertained by using the skin-test in human volunteers. Toxin was prepared by growing Dochez's strain together with the Dick strains for 6 days in Douglas tryptic digest broth enriched by 2% sheep's blood, filtered and tested for sterility in the usual way. On intracutaneous injection in a Dick-positive subject this toxin was found to give a reaction of 35 × 35 mm. in a 1 in 1000 dilution.

Using these a test was set up as in Protocol 1 with tubes of a uniform size, thoroughly mixed and immersed to slightly less than half the depth of their contents in a water-bath at 37° C. At the end of 18 hours no flocculation had occurred. It was thought the failure might be due to the fact that the antitoxin used was in concentrated form, and that the addition of fresh serum might render any possible flocculation present visible. Accordingly 1 part of fresh normal horse-serum was added to 9 of concentrated antitoxin and the tests repeated. At the same time, using the same amounts, two control tests were set up, one containing a mixture of toxin and normal horse-serum, and the other antitoxin and filtered uninoculated 2% blood broth that had been heated slightly to cause hæmolysis. The results of these tests are set out in Protocol 1.

In the foregoing tests the tubes were placed in the water-bath at 11 a.m. and kept at a constant temperature of 37° C. until 6 p.m., when they were removed to the cold room overnight. The following morning they were again placed in the water-bath and inspected every few minutes for appearance of flocculation. This first appeared at the end of 30 hours in the tubes containing 1·2 c.c. toxin and 0·8 c.c. antitoxin. Within a few minutes the range had widened. They were again placed in the cold and read at the end of 50 hours.

The flocculi were large, copious, tended to settle rapidly and were easily dispersed on shaking, leaving an even turbidity.

The control tests were negative.

To further control the experiment a test was set up in the same way as before with a non-toxic filtrate and scarlet fever antitoxin. The non-toxic filtrate was obtained by growing a strain of hæmolytic streptococcus, which had been shown on previous experiments to produce no reaction in human volunteers, in 2% sheep's blood broth for 6 days. The resulting filtrate was tested for the presence of toxin and gave no reaction in a dilution of 1 in 100.

A typical flocculation was obtained as shown in Protocol 2. It differed from the previous one only in that the range had shifted and was narrower.

On repeating the test the temperature of the water-bath accidentally

rose to 50° C., when it was found that a large number of the tubes were flocculated, including the last in the series, which contained filtrate alone. It was thought that the presence of blood products in the filtrate might be responsible for the flocculations obtained, since it was found that when the bath was maintained at 45° C. or over, diffuse flocculation tended to occur in the toxin control. It was accordingly decided to carry on further experiments using non-blood filtrates.

Protocol No. 1.				Protocol No. 2.			
S.F. toxin (c.c.).	S.F. anti-toxin (c.c.).	30 hours.	50 hours.	Non-toxic filtrate (c.c.).	S.F. anti-toxin (c.c.).	30 hours.	50 hours.
0·0	2·0	...	...	0·0	2·0	...	...
0·1	1·9	...	...	0·1	1·9	...	...
0·2	1·8	...	...	0·2	1·8	...	...
0·3	1·7	...	...	0·3	1·7	...	...
0·4	1·6	..	...	0·4	1·6	...	...
0·5	1·5	...	...	0·5	1·5	...	...
0·6	1·4	...	...	0·6	1·4	...	...
0·7	1·3	...	...	0·7	1·3	...	...
0·8	1·2	...	...	0·8	1·2	...	...
0·9	1·1	...	...	0·9	1·1	...	...
1·0	1·0	...	F	1·0	1·0	...	...
1·1	0·9	F	FF	1·1	0·9	...	...
1·2	0·8	FF	FF	1·2	0·8	...	...
1·3	0·7	F	F	1·3	0·7	...	F
1·4	0·6	...	...	1·4	0·6	F	FF
1·5	0·5	...	...	1·5	0·5	FF	FF
1·6	0·4	...	...	1·6	0·4	...	F
1·7	0·3	...	...	1·7	0·3	...	...
1·8	0·2	...	...	1·8	0·2	...	...
1·9	0·1	...	...	1·9	0·1	...	...
2·0	0·0	...	...	2·0	0·0	...	...

F = definite flocculi.
FF = stronger flocculation, and shows tubes in which flocculi first appeared.

Experiments with Non-Blood Filtrates.

These were prepared by growing Dochez's strain and the two Dick strains together in Douglas's tryptic digest broth enriched by 5% ascitic fluid for 6 days. The resultant toxic filtrate was found to give a skin reaction in a positive subject equal to that obtained by the blood-enriched medium.

A test was set up as before, except that the tubes were left in the water-bath at 37° C. until flocculation occurred. Flocculation was not so marked as in the blood broth filtrates and the range was smaller.

As controls, tests were made using the non-toxin producing strain which

had given flocculation in previous experiments and scarlet fever antitoxin, and scarlet fever toxin with normal horse-serum.

In the control containing non-toxic filtrate and antitoxin flocculation occurred in one tube in the series only. The other control remained negative.

The results are shown in Protocol 3:

PROTOCOL No. 3.

Non-blood filtrate (c.c.).	Antitoxin (c.c.).	12 hours.	Non-blood non-toxic filtrate (c.c.).	Antitoxin (c.c.).	12 hours.
0·8	1·2	...	0·8	1·2	...
0·9	1·1	...	0·9	1·1	...
1·0	1·0	...	1·0	1·0	FF
1·1	0·9	...	1·1	0·9	...
1·2	0·8	F	1·2	0·8	...
1·3	0·7	FF	1·3	0·7	...
1·4	0·6	...	1·4	0·6	...
1·5	0·5	...	1·5	0·5	...
1·6	0·4	...	1·6	0·4	...
1·7	0·3	...	1·7	0·3	...

The presence of flocculation in a test containing a non-toxic filtrate seemed to point very strongly to the presence of some reaction other than a toxin-antitoxin one. The fact that antitoxin is usually prepared by injection over a considerable time of large doses of filtrate from organisms grown for a period of several days would almost certainly result in a serum containing antibodies other than antitoxin. It has already been shown by Moloney and Weld (1925), working with diphtheria antitoxin, that this does actually occur. It seemed advisable, then, to discover if the flocculations in our tests were a result of union of toxin-antitoxin alone or a combination of this with a bacterial precipitation. Two methods of procedure were adopted for this purpose—first, by the preparation in rabbits of sera that contained a high titre of precipitin but no antitoxin, and second, by purification of the toxin in an attempt to secure a filtrate free from bacterial antigen.

Preparation of the Non-antitoxic Precipitating Sera.

The Dochez strain, a toxin producing strain from puerperal fever, another from erysepelas, a non-toxin producing strain from erysipelas and a non-toxin producing strain from puerperal fever were selected. These were grown overnight in ascitic broth, thrown down by centrifugalization, washed twice in saline to free the organisms from traces of broth, suspended in saline, heated for 1 hour at 56°C., and injected intravenously weekly in rabbits in increasing doses until trial bleedings showed a high precipitin content in the sera. Agglutinins were in each case found to be present.

Protocol No. 4.

	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
S.F. toxin	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
S.F. antitoxin	0·05	0·1	0·15	0·2	0·25	0·3	0·35	0·4	0·45	0·5	0·55	0·6	0·65	0·7	0·75	0·8	0·85	0·9	0·95	1·0
Saline	0·95	0·9	0·85	0·8	0·75	0·7	0·65	0·6	0·55	0·5	0·45	0·4	0·35	0·3	0·25	0·2	0·15	0·1	0·05	...
50° C. for 15 hours	...	...	...	...	...	...	F	F	2 F	3 F	4 F	4 F	3 F	2 F	F	...	...	...	...	...
S.F. toxin	1·0 in each tube																			
Dochez R.S.	0·05 increasing by 0·05 c.c. per tube to 1 c.c.																			
Saline	0·95 decreasing by 0·05 c.c. per tube to 0 c.c.																			
50° C. for 5 hours	...	...	...	...	...	F	F	F	2 F	3 F	4 F	4 F	4 F	3 F	2 F	F	F	...	...	...
S.F. toxin	1·0 in each tube																			
Toxic strain: Puerperal R.S.	0·05 increasing as before																			
Saline	0·95 decreasing as before																			
50° C for 5 hours	...	...	...	...	...	F	F	2 F	3 F	3 F	4 F	4 F	3 F	3 F	2 F	F	F	...	...	...
S.F. toxin	1·0 in each tube																			
Toxic strain: Erysipelas R.S.	0·05 increasing as before																			
Saline	0·95 decreasing as before																			
50° C. for 5 hours	...	...	...	...	...	...	F	F	F	2 F	3 F	3 F	4 F	3 F	2 F	2 F	F	F	...	...
S.F. toxin	1·0 in each tube																			
Non-toxic strain: Puerperal R.S.	0·05 increasing as before																			
Saline	0·95 decreasing as before																			
50° C. for 5 hours	...	...	...	...	...	...	...	...	F	F	2 F	2 F	3 F	4 F	3 F	F	F	F	...	...
S.F. toxin	1·0 in each tube																			
Non-toxic strain: Erysipelas R.S.	0·05 increasing as before																			
Saline	0·95 decreasing as before																			
50° C. for 5 hours	...	...	...	...	...	...	...	F	F	F	2 F	3 F	3 F	4 F	4 F	3 F	2 F	F	F	...

F = very faint flocculation. Figures (2 F, 3 F, 4 F) denote increasingly heavy flocculation.

The sera were filtered, tested for sterility, and neutralization of toxin experiments carried out in Dick-positive volunteers. They were found to possess no neutralizing value in dilutions of 1 in 5 of the serum.

Tests were set up as follows: 1 c.c. amounts of non-blood scarlatinal toxin were placed in a series of tubes of uniform size. Serum to be tested was added in gradually increasing amounts from 0·05 c.c. to 1·0 c.c. Saline was added to make the volume equal to that in the last tube of the series which contained toxin and serum alone. These were thoroughly mixed and placed in a water-bath at 50°C. until flocculation appeared. Tests were made with the five non-antitoxic rabbit sera and with scarlet fever antitoxin as a control. The results are shown in Protocol 4. It will be seen that a much greater exposure in the water-bath was necessary to bring about flocculation in the antitoxin-toxin mixture than was the case with rabbit sera, and the range of flocculation considerably narrower. A control of toxin and normal rabbit serum was negative.

Experiments to Obtain a Purer Toxin.

For purposes of flocculation experiments in the case of diphtheria it is generally conceded to be desirable to work with strong toxin. It was necessary in our work to keep this fact in view, since the employment of any method of purification which would result at the same time in materially lessening the strength of the toxin seemed inadvisable.

Three methods were investigated: first, rapid growth of the organisms in fluid media, second, rapid growth on solid media, and third, precipitation of toxin by alcohol.

1. *Rapid Growth in Fluid Media.*

Heart broth enriched with 5% sterile ascitic fluid was seeded with a large quantity of freshly grown washed organisms seed incubated for 18 hours. It was then filtered in the usual way. The resultant filtrate was tested for presence of toxin in human volunteers and found to give only weak reactions. It was accordingly considered unsuitable for flocculation experiments.

2. *Rapid Growth on Solid Media.*

The method of Lewis and Henry (1925) were used for this purpose. Roux bottles of serum agar were sown with 2 c.c. of a fresh 18-hour broth culture of Dochez's strain to each bottle and incubated for 18 to 24 hours. At the end of that time the surface growth of each bottle was washed off with 5 c.c. of 0·5% carbol saline. The total amount was filtered, and the sterile filtrate tested by skin reactions. This method was found to yield a toxin which gave a reaction of 45 × 55 mm. in a dilution of 1 in 1000 in a Dick-positive subject, and was considered satisfactory for flocculation tests. The results obtained when this toxin was mixed with scarlet fever antitoxin and with Dochez non-antitoxin rabbit serum are shown in Protocol 5.

It is evident from this that the filtrate contained in addition to toxin a large quantity of bacterial antigen, as shown by the degree of flocculation obtained in the toxin-non-antitoxic serum mixture.

PROTOCOL No. 5.

	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
S.F. toxin . .	1·0 in each tube														
S.F. antitoxin .	0·1	0·15	0·2	0·25	0·3	0·35	0·4	0·45	0·5	0·55	0·6	0·65	0·7	0·75	0·8
Saline . .	To volume of last tube														
15 hours at 50° C.	...	...	...	...	F	2 F	3 F	4 F	3 F	2 F	2 F	F	F	...	...
S.F. toxin . .	1·0 in each tube														
Dochez R.S. .	0·1	0·15	0·2	0·25	0·3	0·35	0·4	0·45	0·5	0·55	0·6	0·65	0·7	0·75	0·8
Saline . .	To volume of last tube														
5 hours at 50° C.	...	...	F	F	F	2 F	3 F	4 F	4 F	3 F	2 F	F	F	...	...

F = very slight flocculation. 3 F = heavier flocculation.
2 F = increased „ 4 F = strongest „

3. *Purification by Alcohol Precipitation.*

The method of Lewis and Henry (1925) was again used. The agar toxin was treated with 6 volumes of absolute alcohol and the resulting precipitate obtained by centrifugalization. This was dried free of alcohol, and made up to the original toxin volume with carbol saline. A certain amount of the precipitate remained insoluble and was separated from the supernatant fluid, which was further treated with 4 volumes of absolute alcohol. This second precipitate was obtained, dried and made up to half the original volume in carbol saline. Most of this precipitate passed into solution, which was obtained in a sterile state by filtration.

On testing the filtrate in human volunteers it was found to have lost considerable toxin, but gave a reaction of 20 × 25 mm. in a dilution of 1 in 1000. Using this filtrate flocculation tests were made with scarlet fever antitoxin and Dochez antibacterial rabbit serum. At the same time a test was set up with undiluted antibacterial rabbit serum and purified toxin. The results are shown in Protocol 6.

From these results it is evident that while purification of the toxin did not result in removing all the antibacterial antigen, only traces were left in combination with toxin, as shown by the test with undiluted Dochez antibacterial serum. It is interesting to note, also, that with removal from the toxin of a large part of the antibacterial antigen, the ability to cause visible flocculations with either antitoxic or non-antitoxic antibacterial serum is removed at the same time. The ability of the purified toxin to cause a satisfactory skin reaction in humans had already been demonstrated.

To further test our hypothesis that the flocculations obtained were mainly due to union of bacteriolytic products in the antigen and antibacterial antibodies in the serum, it was decided to discover if flocculations could be obtained using an extract of the organisms in place of toxic filtrate.

Flocculation Test with Bacterial Extract.

The overnight growth from one litre of plain broth heavily seeded with the Dochez strain was obtained free and washed twice in saline to remove any

Protocol No. 6.

	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Purified S.F. toxin .	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
S.F. antitoxin . .	0·05	0·1	0·15	0·2	0·25	0·3	0·35	0·4	0·45	0·5	0·55	0·6	0·65	0·7	0·75	0·8	0·85	0·9	0·95	1·0
Saline . . .	0·95 decreasing by 0·05 c.c. in each tube.																			
50° C. for 20 hours .									No flocculation											
Purified S.F. toxin .	1·0 in each tube ⟶																			1·0
Dochez antibacterial R.S. . . .	0·05 increasing by 0·05 to 1 c.c. ⟶																			1·0
Saline . . .	0·95 decreasing by 0·05 c.c.																			
50° C. for 20 hours .									No flocculation											
Purified S.F. toxin .	1·0 in each tube																			
Dochez antibacterial R.S. (undiluted) .	0·05 increasing by 0·05 c.c. to 1·0 c.c.																			
50° C. for 5 hours .	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	P	PP	PP

P = very slight clouding at junction of toxin and serum.
PP = heavier " " " "

broth and toxin which might remain on the surface of the organisms. The organisms were mixed with 100 c.c. absolute alcohol, and extracted for 4 hours at boiling-point in a reflux condenser. The extract, freed from organisms, was diluted 1 in 10 in saline, and mixed as in Protocol 4 with scarlet fever antitoxin and Dochez antibacterial serum. As a control a test was made with extract and normal horse-serum. A small amount of flocculation was obtained in the mixture of extract and antitoxin, and a heavier flocculation with antibacterial rabbit-serum. The control remained negative.

To test for the presence of toxin the extract was dried at room temperature overnight, and the deposit made up in 20 c.c. of saline. This was further diluted 1 in 20, and 0·1 c.c. injected intracutaneously in a Dick-positive volunteer. No reaction followed this injection.

DISCUSSION.

A review of the results of this study shows that flocculation occurs when the toxin and antitoxin of scarlet fever are mixed in suitable proportions, but that this flocculation is to any great extent due to the union of toxin and antitoxin is unlikely. It seems quite clear that the preparation of antitoxin by a lengthy course of injections of streptococcus filtrate results in the serum containing a large amount of bacterial antibody in addition to antitoxin. That this bacterial antibody plays an important part in the production of flocculations is already shown when toxic filtrate is mixed with a non-antitoxic antibacterial serum. It seems important, at first, that the range of flocculation this serum produces is considerably wider, and that the period of incubation required to bring about its appearance is so much shorter than with ordinary antitoxin. Two factors may be responsible for this: first, that a relatively greater amount of bacterial protein is injected into the animal in the production of antibacterial rabbit serum than is the case in production of antitoxin; and second, that the concentration of antitoxin has resulted in the loss of a large amount of serum protein that is not entirely restored by the addition of a small quantity of normal horse-serum. That this second factor does play some part is shown by the fact that concentrated serum alone gave negative or uncertain results, but was improved in flocculating power by the addition of fresh serum, and that a sample of unconcentrated antitoxin gave heavier flocculation with a wider range than the concentrated and fresh serum mixture, though its neutralizing value by skin tests was considerably less. Bronfenbrenner and Reichert (1926), working with the toxin and antitoxin of *B. botulinus*, have shown that the flocculation obtained was entirely independent of either the toxic content of the antigen or the antitoxic content of the serum. Moloney and Weld (1925) found that the toxin-antitoxin precipitation in diphtheria bears a close relationship to the agglutinin content of the serum. Though in our own work it was not possible to secure a scarlatinal toxin entirely free from bacterial antigen, there is good proof that the conditions are similar, as shown by the fact that the ability to produce flocculations is directly proportional to the amount of bacterial antigen present in the toxin, and to the amount of bacterial antibody in the serum.

It is not assumed from this work that the flocculation obtained with

diphtheria toxin and antitoxin are to a similar extent due to bacterial antigen and antibody, but rather that in scarlet fever the toxin-antitoxin combination as such does not seem to play a part of any great importance in the flocculation test, and that for this reason the test does not provide us with a suitable method of standardizing the toxin and antitoxin of scarlet fever.

CONCLUSIONS.

1. Flocculation tests have been obtained with scarlet fever toxin and antitoxin.
2. When the filtrate of an atoxic strain is used with antitoxin flocculation is obtained.
3. Animals immunized with washed organisms produce a serum rich in bacterial precipitins but devoid of antitoxin. Tests with this serum and toxin result in typical flocculations.
4. When purified toxin is used the removal of bacterial antigen results in a loss of flocculation power.
5. The presence of flocculation depends on the amount of bacterial antigen in the toxin and the bacterial antibody in the serum.
6. The flocculation test is not a satisfactory means of measuring the toxin and antitoxin in scarlet fever.

The author wishes to thank Prof. J. C. G. Ledingham for his interest and kindly criticism throughout this work.

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